Synthesis and characterisation of a flourinated analogue of NAD+.

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Synthesis and Characterisation of a fluorinated analogue of NAD^+.

Submitted by P J Channon

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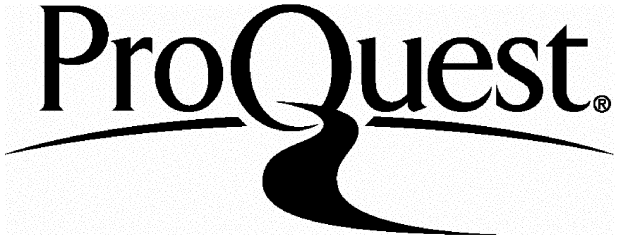

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SUMMARY

Chemical and enzymic routes for the synthesis of a fluorinated analogue of NAD^+ were investigated. 3-Trifluoroacetylpyridine was successfully synthesised chemically and glycosidically linked to 2,3,5-tri-O-benzoyl-D-ribose, but debenzoylation of this product to give the mononucleoside proved impossible.

Enzymic synthesis of 3-trifluoroacetylpyridine adenine dinucleotide from 3-trifluoroacetylpyridine and NAD^+ in the presence of NAD^+ transglycosidase from pig brain was unsuccessful. However, the fluorinated analogue of NAD^+ was prepared by the incubation of 3-trifluoroacetylpyridine, PRPP and ATP in the presence of nicotinamide phosphoribosyl transferase (*Lactobacillus fructosus*) and NAD^+ pyrophosphorylase.

3-Trifluoroacetylpyridine adenine dinucleotide was found to behave as an inhibitor, but not as a substrate in a number of dehydrogenase systems.

ABBREVIATIONS

F-Ura	5-Fluorouracil
F-Urd	5-Fluorouridine
F-UMP	5-Fluorouridine monophosphate
F-UDP	5-Fluorouridine diphosphate
F-UTP	5-Fluorouridine triphosphate
F-RNA	RNA containing 5-fluorouracil
F-dUMP	5-Fluoro-2'-deoxyuridine monophosphate
F-DUrd	5-Fluoro-2'-deoxyuridine
F_3Thd	Trifluorothymidine
F_3TMP	Trifluorothymidine monophosphate
F_3DNA	DNA containing trifluorothymidine
F-dCyd	5-Fluoro-2'-deoxycytidine
d-UMP	Deoxyuridine monophosphate
TMP	Thymidine monophosphate
DMSO	Dimethyl sulphoxide
NADH	NAD (reduced form)
ADPR	Adenosine-5'-diphosphate riboside
NAD^+	Nicotinamide adenine dinucleotide
RNA	Ribonucleic acid
t-RNA	transfer Ribonucleic acid
DNA	Deoxyribonucleic acid
ATP	Adenosine-5'-triphosphate
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
NMN	Nicotinamide mononucleotide
T.C.A.	Tricarboxylic acid
IR.	Infra red

n.m.r.	Nuclear magnetic resonance
U.V.	Ultra violet
g.l.c.	gas liquid chromatography
t.l.c.	Thin layer chromatography
eV.	electron volts
p.p.m.	parts per million
Hz.	Hertz
m.p.	melting point
b.p.	boiling point
c.p.m.	counts per minute

INTRODUCTION

Fluorinated analogues of natural substrates in which either a hydrogen atom or a hydroxy group in the original compound is replaced by fluorine have, in the past, proved to be of considerable value in the elucidation of metabolic pathways, and as a probe at the active sites of enzymes. In view of the wide involvement of NAD^+ in enzymically catalysed oxidation-reduction reactions a fluorinated analogue of this coenzyme could be of great use as a probe, particularly now that the techniques of ^{19}F n.m.r. spectroscopy have been applied to enzyme systems.

In view of the direct involvement of the nicotinamide mioety in the oxidation-reduction mechanisms involving NAD^+, it was decided to introduce fluorine, in the form of a trifluoromethyl group, into the nicotinamide component. Both chemical and enzymic approaches to such a synthesis are possible, and the methods available will be reviewed later in the present Introduction.

BIOLOGICAL ACTIVITY OF FLUORO-COMPOUNDS.

(a) Analogues in which fluorine replaces hydrogen.

Although the study of organic fluorine compounds began in 1896 when Swarts described the chemistry of a series of fluoroacetates it was not until much later (Schrader et. al.; 1946) that the toxicity of these compounds, and their potential as insecticides and rodenticides became appreciated. Marais (1944) observed that fluoroacetate is the major compound present in the South African plant Dichapetalum cymosum, and was so able to explain the sickness and death of cattle which grazed on pastures containing these plants. In view of the economic implications of this finding research into the toxicity and metabolism of fluoroacetate was initiated and pursued in the following

years.

The toxicity of fluoroacetate varies considerably from system to system, for instance, a dose of 60 µg/Kg is often sufficient to kill a dog, whereas the South African clawed toad can survive a dose of 500 mg/Kg (Quin and Clark; 1947), and fluoroacetate has been shown to have little effect on microorganisms (Kalnitsky and Barron; 1947) or on isolated enzymes (Bartlett and Barron: 1947).

Fluoroacetate was found to competitively inhibit acetate metabolism in Baker's yeast (Saccharomyces cerevisiae) and in Corynebacterium creatinorans, both of which microorganisms utilise acetate rapidly (Kalnitsky and Barron; 1947). Martius (1949) and Liebecq and Peters (1949) in simultaneous experiments showed that fluoroacetate was in some way interferring with citrate metabolism. Buffa and Peters (1949) showed shortly afterwards that fluorocitrate arrested the T.C.A. cycle and caused citrate accumulation in vivo in rat and guinea pig, but had no effect on isolated enzyme fractions. Since this time it has been suggested that the inhibitary substance isolated from tissue homogenates after respiration in the presence of fluoroacetate is monofluorocitrate. Thus, synthetic samples of monofluorocitrate were shown to have a comparable I.R. spectrum to that of a fluorinated tricarboxylic acid isolated by Peters et. al. (1953 a and b) from kidney tissue which had been incubated with monofluoroacetic acid.

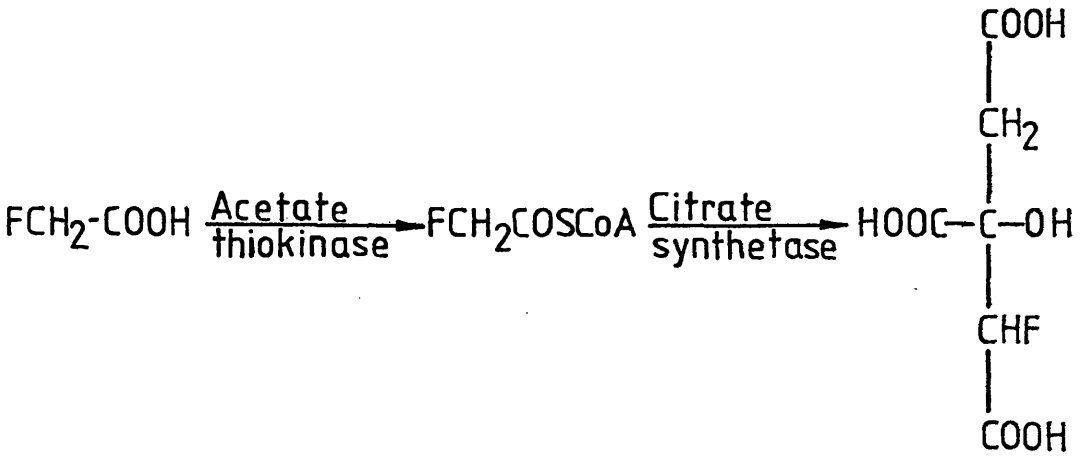
Lotspeich et. al. (1952) demonstrated that the 'inhibitor fraction' isolated after incubation of kidney homogenates with fluoroacetate would inhibit aconitase preparations, thus implicating this enzyme

as a possible point at which the 'biochemical lesion' occurs after fluoroacetate poisoning. Peters (1961) has demonstrated as much as 50% inhibition of aconitase by as little as 10^{-8} to 10^{-9}M of enzymically prepared fluorocitrate, and has described the production of the toxic fluorocitrate from the non-toxic precursor, fluoroacetate, as "lethal synthesis". (Fig. 1)

Monofluorocitric acid has been synthesised chemically (Rivett; 1953: Fanshier et. al.; 1964) and has been shown to consist of two pairs of enantiomers (Fig. 2) giving two spots on electrophoresis, one corresponding to the erytho pair (I and III) and the other to the threo pair (II and IV). On the basis of X-ray crystallographic studies Carrell et. al. (1970) assigned the slower moving (more acidic) spot to the threo configuration.

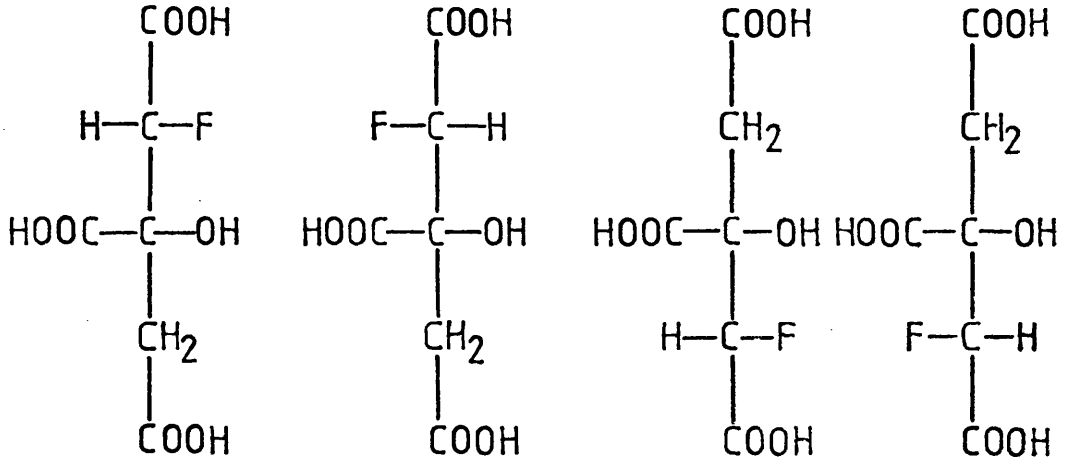
Fanshier et. al. (1964) showed that citrate synthetase catalyses the condensation of fluoroacetyl CoA with oxaloacetate to give an isomer of fluorocitrate which strongly inhibits aconitase, whereas the isomer formed when acetyl CoA is condensed with fluorooxaloacetate does not inhibit aconitase. Both of these isomers run as the slower spot on electrophoresis and therefore have the threo configurations. The isomers with the erythro configuration were found not to be inhibitors of aconitase. If it is assumed that citrate synthetase shows the same stereo specificity to fluoroacetyl CoA as it does to acetyl CoA then the inhibitory isomer must have the structure III or IV, which therefore leaves structure IV. The result of the lethal synthesis of fluorocitric acid from fluoroacetic acid is accordingly (-) - threo monofluorocitrate (IV) which blocks the T.C.A. cycle by inhibition of aconitase.

Fig. 1.



Biosynthesis of fluorocitrate.

Fig. 2.



I Erythro (2R3S)

II Threo (2S3S)

III Erythro (2S3R)

IV Threo (2R3R)

Enantiomers of monofluorocitric acid.

Other fluorinated metabolites of monofluoroacetate have been postulated. Miura and Hori (1961) reported evidence of a fluorinated ketone and Schaefer and Machleidt (1971) published evidence of the conversion of fluoroacetate to amino-acids in various mammalian tissues.

As work on the elucidation of the mechanism of fluoroacetate toxicity was progressing, it became apparent that other fluorinated aliphatic compounds were also toxic. Various groups of workers (Saunders; 1947: Buckle et. al. 1949: Pattison and Saunders; 1949: Pattison; 1959) suggested that long-chain ω-fluoro fatty acids with an even number of carbon atoms would be expected to be highly toxic whereas those with an odd number of carbon atoms would be relatively non-toxic. This is compatible with the β-oxidation mechanism accepted for fatty acid degradation: the even numbered acids passing through a highly toxic fluoroacetate stage and the odd numbered acids to the relatively non-toxic β-fluoropropionate. This rule was also applied to ω-fluoronitriles by Pattison et. al. (1956) who found that those compounds with an odd number of carbon atoms were very toxic, whereas those with an even number were not. This was taken as indicating biodegradation via carbon-cyanide cleavage, rather than by a reduction of the $-C \equiv N$ bond.

As a result of metabolic and enzymic studies on these various naturally occuring fluorine compounds it became evident that synthetic fluorine containing compounds could be of use in the elucidation of the mechanism of many biological systems.

β-Fluoropyruvic acid, a compound of potential biological interest, was first synthesised by Mager and Blank (1954) who reported growth inhibition of Escherishia coli and Aerobacter aerogenes at low concentrations of the fluoroanalogue. It was also shown to be toxic to rats at higher levels, causing death without the high level of citrate accumulation usually associated with fluoro-acetate poisoning. Subsequently Gal et. al. (1956 a, b) reported that in the rat, a fatal dose (ca. 80 mg/Kg) of β-fluoropyruvate reduced the amount of ^{14}C-carbon dioxide expired from ^{14}C-acetate in vivo, and lowered the level of pyruvate in the blood, indicating that pyruvate utilisation was not blocked. Some in vitro work with kidney particle preparations showed that β-fluoropyruvate blocked the formation of citrate from fumarate, but did not inhibit the disappearance of citrate, the inference being that aconitase was not inhibited and hence that fluorocitrate was not formed in appreciable quantities. β-Fluoropyruvate has a powerful effect on the enzymes of pyruvate metabolism and was shown by Avi-Dor and Mager (1956a) to exercise its metabolic effect by behaving in a similar manner to iodoacetate, which blocks protein or enzyme -SH groups. This reaction was shown (Peters and Hall; 1957) to be accompanied by the splitting off of the fluorine atom,

$$R\text{—}SH + FCH_2COCOOH \rightarrow RSCH_2COCOOH + HF$$

and it may be that β-fluoropyruvate could have a use in -SH group estimation (Avi-Dor and Mager; 1956 b).

One rationale behind the use of fluorine for the production of isosteres having potential biological or pharmacological importance has been its similarity in size to a hydrogen atom (Fig. 3). Thus, in the laboratories of both Bergman (1961) and Kun (Kun and Dummel;

Fig.3.

Parameters of various elements.

Element	Bond length[a]	Van der Waals radius[b]	Electronegativity[c]
H	1.09	1.20	2.1
O(in OH)	1.43	1.40	3.5
F	1.39	1.35	4.0
Cl	1.77	1.80	3.0
Br	1.94	1.95	2.8
I	2.1 - 2.2	2.15	2.5
S (in SH)	1.82	1.85	2.5

a) Tables of interatomic distances and configuration in molecules and ions, The Chemical Society (London). Special publication No. 18. Ed. L.E. Sutton (1965).

b) The nature of the chemical bond. L. Pauling. 3rd Edition, Cornell University Press, Ithaca, New York, p260.

c) Ibid. p.90

1969) a concentrated effort has been made to synthesise intermediates of the T.C.A. cycle and some related amino acids in which fluorine replaces a hydrogen atom. The effect of many of these compounds is not dramatic at a metabolic level, although some appear to be specific inhibitors of isolated enzyme reactions. Thus, monofluorooxaloacetate has been found to interact with a number of enzymes for which oxaloacetate is a substrate (Kun; 1969) while fluoroacetate is a potent inhibitor of malate dehydrogenase ($K_i < K_m$ for oxaloacetate) implying the reduction of oxaloacetate and difluorooxaloacetate (also a substrate for this enzyme) in mitochondria (liver and kidney) and cytoplasm (kidney but not liver) (Kun and Volfin; 1966 a). It has been suggested that differential inhibition of this type may result from selective recognition of inhibitors by isozymes possessing differing catalytic properties. Such tissue specific differences may be a relatively common phenomenum. Thus oxidation of succinate by liver and kidney mitochondria is unaffected by monofluorooxaloacetate even at a concentration of 0.2 mM, whereas in heart mitochondria, under similar conditions succinate oxidation is abolished (Kun and Volfin; 1966 b).

Organ-specific differences were also demonstrated in the effects of monofluorooxaloacetate on the respiration of mitochondria (using oxaloacetate and pyruvate) from brain, liver and kidney in which the addition of 0.33 mM monofluorooxaloacetate caused 80 to 90% inhibition of oxygen uptake after 30 minutes (Fanshier et. al.; 1962). Citrate levels were diminished to approximately 50% in liver and kidney, but almost unaffected in brain mitochondria. The potent inhibition of oxygen uptake, and lesser inhibitory effects on citrate levels in brain could be explained in terms of the demonstrated

weak *in vitro* effect of fluorooxaloacetate on citrate formation from acetyl CoA and oxaloacetate coupled with the more pronounced effect on malate dehydrogenase. Similar experiments, performed under initial rate conditions in an oxygen electrode showed that the fluoro-acid did not initially affect oxygen uptake and indicated a time dependent adjustment of mitochondrial metabolism (Kun and Volfin; 1966 b).

An advantage of using fluoroanalogues is that their action is likely to be related to the function of specific enzymes. Kun (1969) suggests that fluoroanalogues in combination , for example, with uncoupling agents, may be able to discriminate between energy coupled and substrate level control mechanisms.

Fluoroanalogues can disrupt or modify cellular functions in a variety of ways. Incorporation of the analogue into cellular macromolecules has now been demonstrated in several systems and analogues already shown to be active in this respect include the fluorinated amino-acids 5,5,5-trifluoroleucine , 2,3- and 4-fluorophenylalanine, 3-fluorotyrosine and 4-fluoroproline (Halvorson and Speigelman; 1952: Munier and Cohen; 1959: Rennert and Anker; 1963 Richmond; 1963: Gottlieb *et. al.*; 1965: Munier and Sarrazin; 1964: 1966).

p-Fluorophenylalanine has been shown to prevent mature polio virus formation (Leventas *et. al.*; 1962) while still permitting synthesis of infectious RNA. The fluoroanalogue does not necessarily inhibit protein synthesis, but may act by virtue of its incorporation into a protein which becomes functionally impaired. Thus, in the presence of p-fluorophenylalanine, viral coat protein is either not formed, is formed but is functionally inadequate, or the maturation process is sensitive to the fluoroanalogue.

Fluoroanalogues sometimes reveal aspects of biochemistry which are

not easily detected by a non-analogue approach. Tryptic digests of the alkaline phosphatase from E. coli yield six peptides that contain phenylalanine (Richmond; 1963), four of which contain one phenylalanine molecule in the chain, while the others contain two. Examination of the enzyme after biosynthetic labelling with ^{14}C-p-fluorophenylalanine showed that all the peptides which originally contained phenylalanine now contained the fluoroanalogue, demonstrating that all the phenylalanine residues in alkaline phosphatase are accessible to replacement by the fluoroanalogue. The degree of replacement under conditions of direct competition between p-fluorophenylalanine and phenylalanine shows no variation in the ratio of the two amino-acids incorporated into any of the six peptides, indicating that the system responsible for the incorporation of phenylalanine into various positions in alkaline phosphatase may be equivalent. Arnstein and Richmond (1964) demonstrated that this same fluoroanalogue was incorporated into protein by a cell free system from rabbit reticulocytes in a quantitative manner similar to phenylalanine. They found that the incorporation of both phenylalanine and p-fluorophenylalanine is stimulated to approximately the same extent by the addition of polyuridylic acid to the reticulocyte system, indicating that at least one code, the UUU triplet, for phenylalanine does not discriminate against the p-fluoroanalogue. If there is any degeneracy in the code for phenylalanine in the reticulocyte system the various t-RNA's must have similar specificities for phenylalanine and p-fluorophenylalanine.

Non mucoid strains of E. coli produce capsules and yield mucoid colonies when grown in the presence of p-fluorophenylalanine (Kang and Markovitz ; 1967 a, b). The organism reverts to the

normal incapsulated state when subcultured in media free of the fluoroanalogue or when phenylalanine is added to depress its utilisation. Analysis revealed elevated levels of certain enzymes concerned with polysaccharide synthesis when the fluoro-analogue was present in the medium. Kang and Markovitz suggest that synthesis of these enzymes has been de-repressed, possibly because p-fluorophenylalanine has been incorporated into the regulator protein coded for by the relevent regulator gene(s) of the organism, leading to the synthesis of ineffective repressor molecules. A report by Rennert (1969) has appeared which suggests that the formation of leucine - t - RNA and trifluoroleucine - t - RNA during the embryonic development of the mouse is a function of gestational stage. Results indicate the presence of a leucine - t - RNA synthetase in unborn mice of gestational age less than ten days which is capable of acylating t - RNA with trifluoroleucine almost to the same extent as leucine. A second leucine - t - RNA synthetase, similar chemically and physically to the enzyme from a more mature embryonic tissue has been isolated from this embryonic tissue, as well as adult tissue. Trifluoroleucine - t - RNA was not formed if the enzyme and t - RNA were obtained from late embryonic tissue (gestational age greater than fourteen days), newborn mice, or adult organs. This investigation revealed a unique enzyme, a leucine - t - RNA synthetase, present in early embryonic tissue which disappears during the process of embryogenesis and is absent from adult systems.

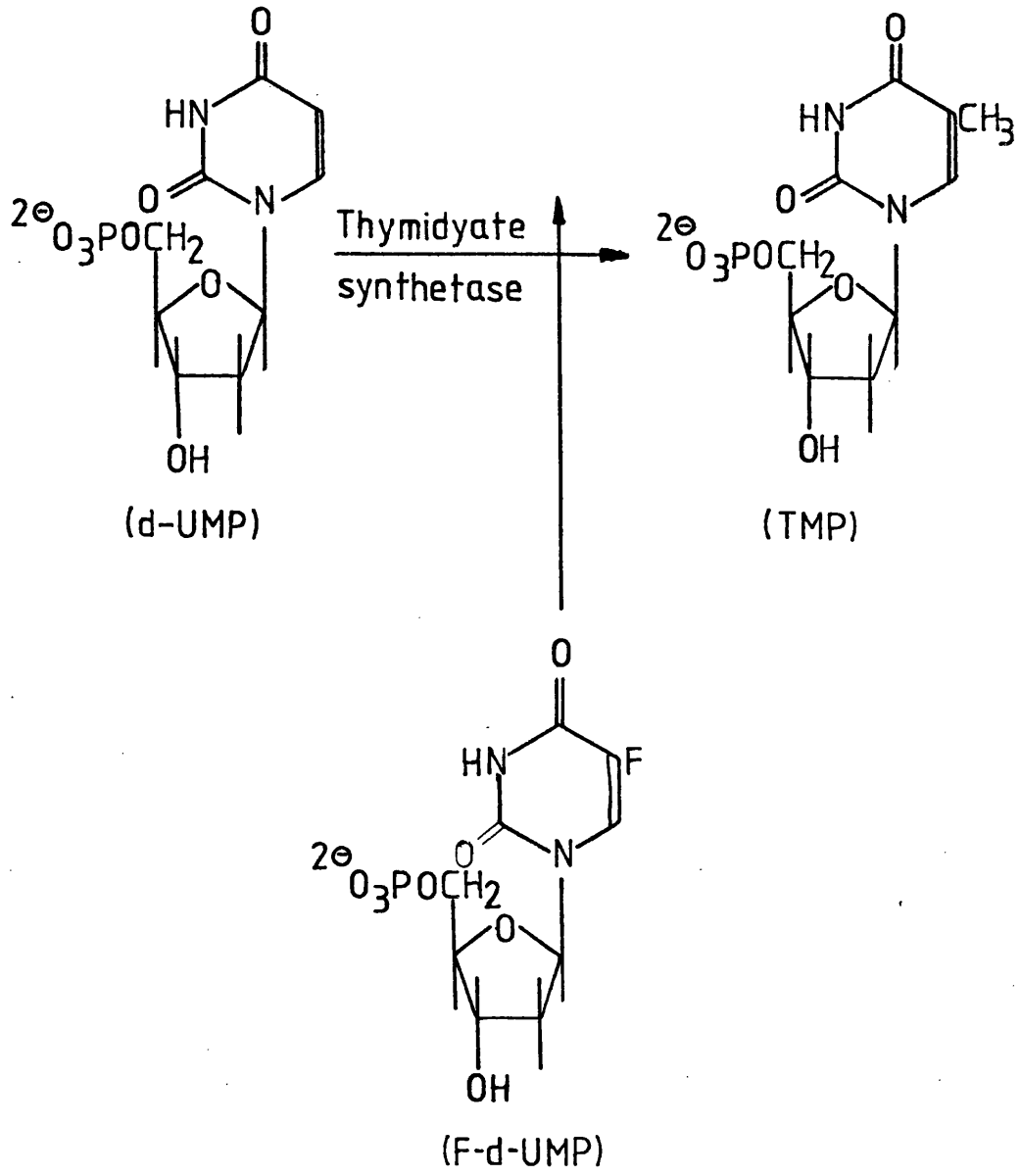
The rewarding results obtained with fluoroacetate and its derivatives have motivated many workers, in both biological and medicinal chemistry to examine the biochemical and therapeutic effects of natural products in which a hydrogen is replaced by a fluorine. In the field of nucleic acid research, many fluorinated purine and pyrimidine

nucleotide analogues have been synthesised (Heidelberger; 1965: Hartmann and Heidelberger; 1961: Reyes and Heidelberger; 1965: Heidelberger; 1972: Wright et. al; 1969: Wright et. al.; 1970). Some of these compounds display a type of biological activity which renders them useful as anti-tumour agents. It has been known for some time that certain tumours utilise uracil for nucleic acid biosynthesis to a greater extent than most normal cells (Rutman et. al.; 1954). In an attempt to exploit this and the fact that most cancer cells differentiate more rapidly than normal cells, Heidelberger and his co-workers (1965) have synthesised fluorinated uracil analogues which could potentially interfere with nucleic acid biosynthesis in cancer cells. Of the compounds synthesised, some of the most useful chemotherapeutic agents are 5-fluorouracil and 5-fluoro-2′-deoxyuridine, the former being possibly the best anti-cancer drug currently available. Both compounds are themselves inactive and have been shown to require a metabolic transformation within the cell to an 'active' form (5-fluoro-2-deoxyuridine monophosphate). This compound is a potent competitive inhibitor of thymidylate synthetase (K_i = 2.2 x 10^{-8}M) (Hartmann and Heidelberger; 1961) which is the enzyme responsible for the conversion of 2′-deoxyuridine monophosphate into 2-deoxythymidine monophosphate. This, in the terminology of Peters, can be considered as another example of 'lethal synthesis'. Both 5-fluorouracil and 5-fluoro-2′-deoxyuridine are thought to be converted to 5-fluoro-2′-deoxyuridine monophosphate via pathways normally associated with the synthesis of 2′-deoxythymidine monophosphate. On reaching the 5-fluoro-2′-deoxyuridine monophosphate stage, the compound is not metabolised further, and having a high apparent affinity for thymidylate synthetase,

inhibits this enzyme, and consequently nucleic acid biosynthesis (Fig. 4).

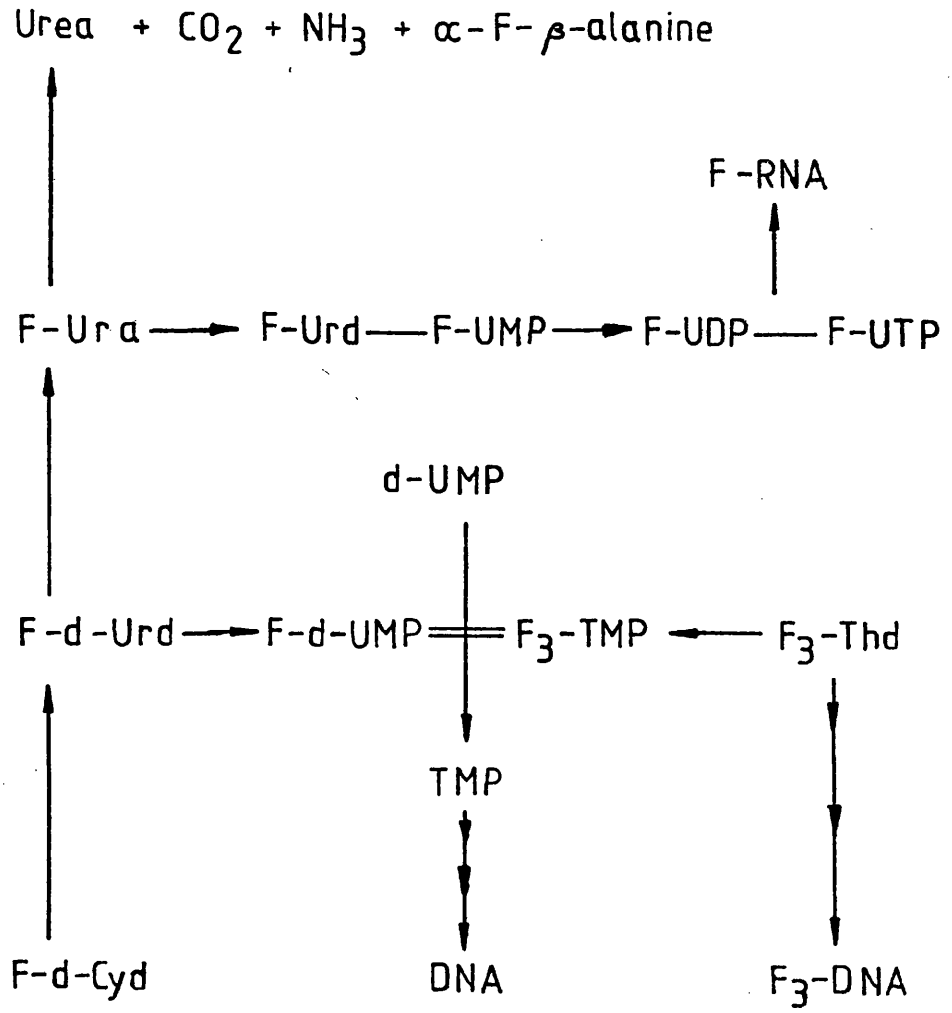
Considerable evidence now exists that thymidylate synthetase inhibition is the major antitumour mechanism of fluorouracil although the incorporation of the analogue into RNA cannot be completely eliminated (Heidelberger; 1972). An advantage of using 5-fluorouracil and 5-fluorouracil-2'-deoxyuridine for chemotherapeutic purposes is that they are degraded in animals to the relatively non-toxic α-fluoro-β-alanine rather than a fluoro compound possessing other toxic properties (Fig. 5) (Chaudhuri et. al.; 1958). The pyrimidine analogue trifluorothymine is not significantly active in mammalian systems. The nucleoside of this compound, trifluorothymidine, was found to be highly active against tumours and DNA viruses. 5-Trifluoromethyl-2'- deoxyuridine monophosphate inhibits thymidylate synthetase in a competitive manner if added simultaneously with the substrate (Reyes and Heidelberger; 1965), but non-competitively if the enzyme is preincubated with the inhibitor. This may mean that an alkylation has taken place near or at the active site. Fridland et. al. (1971) have now affected a 4700-fold purification of the enzyme from Ehrlich ascites carcinoma cells in order to elucidate whether or not an alkylation does take place. That thymidylate synthetase is the locus of antitumour activity of the nucleoside trifluorothymidine is suggested by the fact that very little of the fluoro-analogue appears in the DNA of mammalian cells. In the case of viruses such as the T 4 B bacteriophage, however, as much as 10% of the thymine in the DNA can be replaced by the trifluoroanalogues which results in a mutagenic response (Gottschling and Heidelberger; 1963). In

Fig. 4.



Inhibition of thymidylate synthetase by 5-fluoro-2'-deoxyuridine monophosphate

Fig. 5

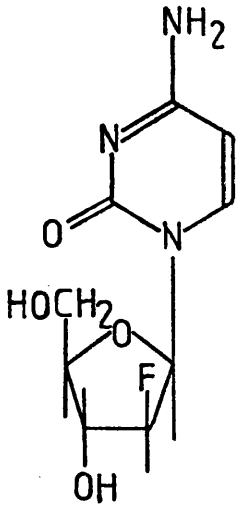


Degradation of 5-fluorouracil and 5-fluoro-2'-deoxyuridine in animals.

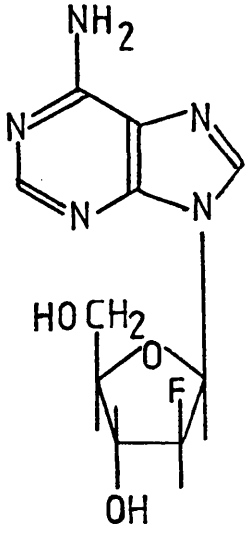
pursuing the antiviral properties of trifluorothymidine Kaufman and Heidelberger (1964) found this compound to be extremely active against Herpes simplex keratitis of the rabbit cornea, being more potent in this respect than any other analogue previously used for therapeutic purposes (Kaufmann; 1965). To study the mechanism of antiviral action of this compound, Heidelberger and his co-workers have established a standard system for examining the effect of trifluorothymidine on Vaccina virus replicating in HeLa cells. The fluoroanalogue was ten times more active as an antiviral agent in this system than any other compound (Umeda & Heidelberger; 1969) and the effect was irreversible, even after a delayed thymidine administration. That this compound was actually incorporated into viral DNA was eventually demonstrated by sucrose gradient centrifugation experiments (Fujiwara and Heidelberger; 1970). Viral particles, after incorporation of trifluorothymidine into their DNA were completely non-infective, producing abnormal m-RNA (Oki and Heidelberger; 1971). Studies are continuing in Heidelberger's laboratory in an attempt to elucidate the complete mechanism of antiviral action of trifluorothymidine.

The antitumour approach that is being used by Fox and his co-workers (Wright et. al.; 1969: 1970) is to synthesise nucleosides bearing fluorine in the sugar moiety rather than in the base (as Heidelberger did). They are, however, continually harassed by deaminase activity. 9-(2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl)adenine(Fig. 6) (Wright et. al.; 1969), a potential inhibitor of nucleic acid biosynthesis, is rapidly metabolised to the inactive cytotoxic agent (Wright et. al.; 1970). The pyrimidine analogue 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl) cytosine (Fig. 6) appears to be deaminated relatively slowly, and

Fig. 6



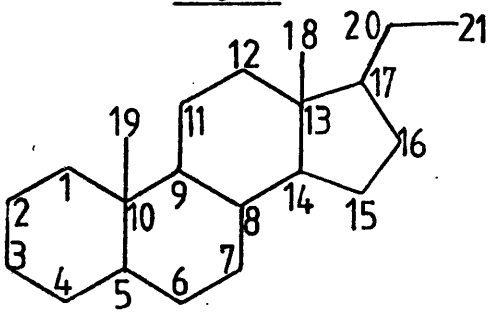
1-(2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl)cytosine.



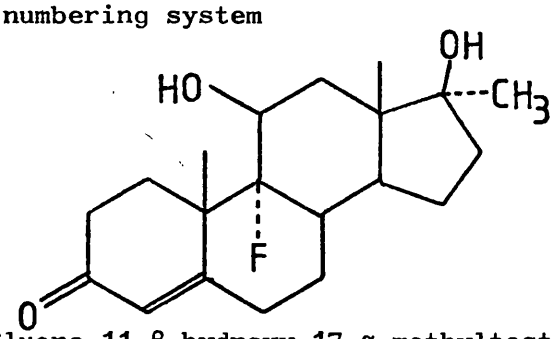
9-(2'-Deoxy-2'-fluoro-β-D-arabinofuranosyl)adenine.

screening studies reveal that this compound is active against suspensions of L1210 leukemia cells (Wright et. al.; 1970). Fluorination of steroid molocules frequently produces analogues exhibiting enhanced biological activity over that of the parent compound (Burger; 1970: Chen and Borrevang; 1970: Wettstein; 1972). Such analogues have many potential uses, for example as oral contraceptives, anti-inflammatory drugs and in the treatment of hormonal sexual disorders and certain types of cancer. The androgen, 9 α-fluoro-11 β-hydroxyl-17 α-methyltestosterone (Fig. 7a) shows high androgenic and anabolic activities. These properties, coupled with its relatively weak action on the hypophysis render it useful for the treatment of certain hypogonad disorders and breast cancers (Talley et. al.; 1964). Some 'prototype' steroids possess more than one enhanced activity and this is undesirable in certain instances. Both the glucocorticoid and mineralocorticoid activities of 9 α-substituted cortisols are increased in relation to the parent hormone cortisol (Fig. 7b). Further substitution, for example of a fluorine atom into the 6 α-position, increases the glucogenic and anti-inflammatory activity of the compound whilst diminishing possibly unwanted sodium retaining properties (Fig. 8). Fluorination of the steroid nucleus in the 6α, 6α and 9α, 16α, 6α and 16α, and 6α, 9α and 16α positions has proven most effective in changing a variety of activities normally associated with the parent hormone. In this connection, the 6α, 9α and 16α fluoro series of prednisolone acetates are potent as anti-inflammatory and thymolytic agents (Fig. 5c) (Magerlein et. al.; 1960: 1964; Bowers et. al; 1959).

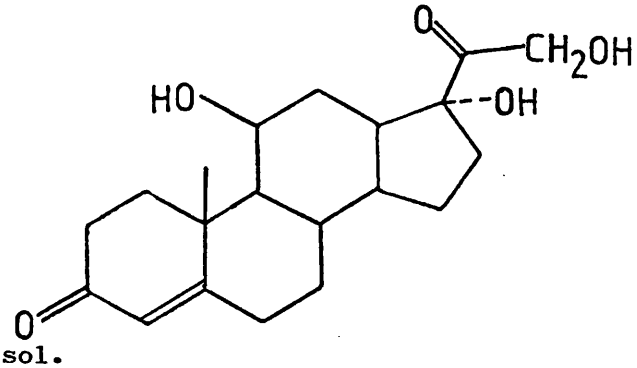
Fig. 7



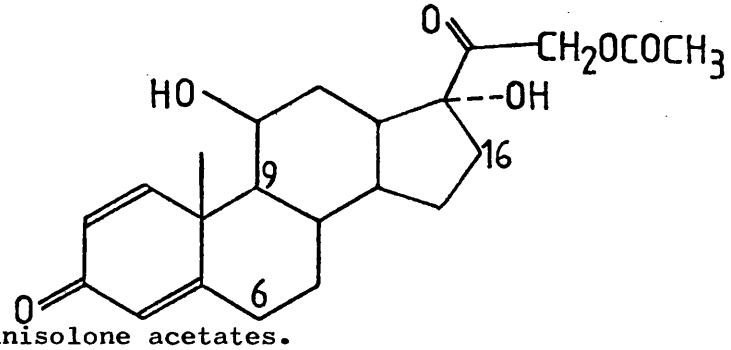
Steroid numbering system



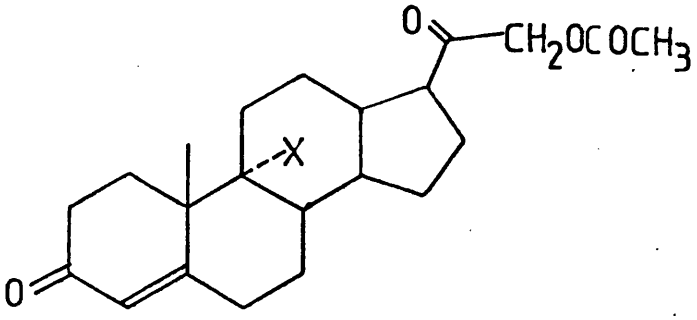
(a)9 α-Fluoro-11 β-hydroxy-17 α-methyltestosterone.



(b)Cortisol.



(c)Prednisolone acetates.



(d)(X=F);9 α-Fluoro-11 α-deoxycorticosterone acetate.
(e)(X=H);11 α-deoxycorticosterone acetate.

Fig. 8.

Effect of fluorination in different positions of the cortisol molecule. (Wittstein;1972.)

Position of fluoro-substituent	Change in activity. Glucocorticoid	Anti-inflammatory	Mineralocorticoid
9	++	++	+++
9 (No 11-OH)	--		+++
12 (17-OH blocked)	++	++	+++
6	++	++	--
6 +9	+++	+++	+
2	-	-	
4		--	
15		+	--
15+19		++	--
16	++	++	
16 +6	+++	+++	
16+6+9 (F in place of -OH)		++++	---
11		--	
17	--		
21	--	--	---

It is still not possible to predict the biological effect of structural modifications in steriod molecules. Fried and Borman (1958) have proposed an explanation for the enhancement of glycogenic action observed with 9α-substituted cortisoles. Although, in general, the smaller the 9α-substituent, the greater the effect, steric factors could not account completely for the results obtained with all the compounds tested. For example, the effects of 9 α-hydroxyl substitution were some fifty times less than that of the fluoro-analogue, although the groups are of equivalent size. It was therefore proposed that the electronegativity of the atoms substituted at the 9α position would, by virtue of its electron withdrawing properties, increase the acidity of the 11 β hydroxyl group, possibly strengthening a hydrogen bond between this group and the receptor molecule.

9 α-fluoro-11α-deoxycorticosterone acetate (Fig. 7d) (Bergstrom and Dodson; 1960) was found to have twelve times the sodium retaining properties of 11α-deoxycorticosterone acetate (Fig. 7e) (Kagawa and Jacobs; 1960) Bergstom and Dodson (1960) have challenged Fried and Borman's interpretation of the influence of the 9 α substituent on 11α-oxygenated steroids (9 α-fluoro-11 α-deoxycorticosterone acetate has no 11 α-oxygen function) and have suggested that the 9 α-fluoro group possibly interferes with metabolic 9-hydroxylation reaction. This pathway is not, however, thought to be important in mammals. It is possible that the sodium-retaining properties, also observed with some 9α-fluoro-11 β-hydroxyl steroids, are due to a mechanism similar to the one underlying enhanced 9α-fluoro-11-deoxy-corticosterone activity. A complete analysis of this problem cannot, however be sought until the exact nature of the receptors for various hormonal activities has been determined. Chemical evidence (Eschenmoser;1964) indicates a slower chromic acid oxidation of 9α-fluoro-11 β-hydroxy

steroids than of the parent compounds, and he suggests that the hydroxyl group is stabilised by the negative inductive affect of the fluoro-atoms. Bush and Mahesh (1964) predicted, from the evidence of Eschenmoser, that, in vivo, the ketosteroid (inactive)/hydroxy steroid (active) ratio will be diminished, thus effecting greater glucocorticoid activity, although they also suggested the possibility of competitive inhibition of the 11-carbon oxidoreductase reaction by the fluoro-analogue. Bush et. al. (1968) subsequently isolated in 11 β-hydroxy steroid dehydrogenase from rat and guinea pig liver microsomes. 9 α-Fluorocortisol was a competitive inhibitor of enzymic cortisal oxidation, but is itself reduced at 5-10 times the initial velocity of cortisone.

Fluorinated analogues can be used to help elucidate the mechanism of action and active sites of individual enzymes. Difluoro-oxaloacetate has been used in this way to study aspartate transaminase (Briley et. al.; 1977 a:b). This will be discussed in more detail later.

(b) Analogues in which fluorine replaces a hydroxyl group.

It has already been suggested (see p. 5) that the biological activity elicited by isosteres of natural compounds in which a fluorine atom replaces a hydrogen site results from the similarity in size between a fluorine and a hydrogen. It can also be seen (Fig. 3) that fluorine and oxygen (in, for example, a hydroxyl group) have similar bond lengths to carbon, similar Van der Waals radius and comparable electronegativity. Moreover it has been demonstrated that covalently-bound fluorine, like a hydroxy group can accept a hydrogen bond. (Buckley et. al.; 1968: Martin; 1965: McDaniel and Brown; 1955: Pavlath and Leffler; 1962). These physico-chemical factors have been implicated in an explanation of the close resemblance in the crystal structure between 2-deoxy-2-fluoro-erythritol and erythritol itself (Bekoe and Powell; 1959), as determined by X-ray crystallographic measurements.

It is only in the last thirty years that analogues have become available

in which fluorine replaces a hydroxy group, and hence the ability of fluorine to mimic hydroxyl groups in biological systems has only recently been examined.

1-Deoxy-1-fluoro-D-glycerol and 1-deoxy-1-fluoro-L-glycerol were synthesised stereospecifically using independent routes from D-mannitol (Lloyd and Harrison; 1971: 1973). The enantiomers were also interconverted by a route which was also used to prepare 1-deoxy-D-glycerol from its enantiomer 1-deoxy-L-glycerol.

Together with 2-deoxy-2-fluoroglycerol and propan-1, 3-diol (2-deoxyglycerol) the above compounds constitute a series of analogues in which each hydroxyl group of glycerol is successively replaced by fluorine or hydrogen. These analogues were examined as inhibitors or substrates of glycerol kinase (Eisenthal et. al.; 1972) using steady state kinetics. The behaviour of 1-deoxy-1-fluoro-sn-glycerol as a substrate and 3-deoxy-3-fluoro-sn-glycerol as a competitive inhibitor allowed a reasonably precise location of the fluorine atoms within the enzymes active site. However, distinction between the two alternative orientations of the fluorine atom at C-2 could not be made on the kinetic data alone. However, it was later shown (Briley et. al.; 1975) that glycerol kinase catalyses the phosphorylation of the symmetrical substrate 2-deoxy-2-fluoroglycerol by ATP to an assymetric product, 2-deoxy-2-fluoro-sn-glycerol-3-phosphate.

This conclusion was based on the nmr. spectroscopic data obtained for chemically prepared racemic 2-deoxy-2-fluoroglycerol phosphate. The signals for the methylene groups at the phosphorylated and unphosphorylated ends of the molecule are well defined and by a study of the splitting patterns and comparison with the spectra of the synthetic precursors these signals were assigned to the appropriate enantiomers.

Briley et. al. also chemically synthesised 2-deoxy-2-fluoro-sn-glycerol labelled at C-1 with deuterium, enzym - ically phosphorylated this compound using glycerol kinase, and purified and isolated the crystalline product using ion-exchange chromatography. On the basis of the nmr spectroscopic data the phosphate group in this compound was assigned to the non-deuterated end of the molecule.

Phosphorylation of the monodeoxy-and monodeoxy-monofluoroglycerols gave a series of analogues of sn-glycerol-3-phosphate. These analogues were investigated as substrates or inhibitors of α-glycerophosphate dehydrogenase from intact and solublised flight muscle mitochondria from Locusta migratoria. (Lloyd and Harrison; 1974). sn-Glycerol-3-phosphate and its analogues in which the C-1 hydroxyl group was replaced by either hydrogen or fluorine all exhibited substrate activity and Michaelis-Menton kinetics, whilst substrate analogues in which the C-2 hydroxyl group is in the D-configuration or has been replaced by hydrogen or fluorine lack substrate activity but behave as competitive inhibitors of the oxidation of sn-glycerol-3-phosphate using either the intact or solublised mitochondrial enzyme preparations. The C-1 and C-2 deoxyfluoro analogues of sn-glycerol-3-phosphate and of its enantiomer have similar kinetic parameters to the corresponding deoxy analogue in both enzyme preparations. The conclusions from these experiments using monofluoro monodeoxy glycerols and their phosphates were that, whereas replacement of a hydroxy group by fluorine led to an enzyme-substrate analogue complex closely mimicking the unfluorinated hydroxy containing complex, the kinetics of the fluoro-analogue were similar to those of the deoxy analogue rather than those of the original substrate.

The majority of synthetic analogues in which fluorine replaces a hydroxyl group are monodeoxymono-fluoro sugars and their related compounds. Among sugars that have already been reported are the complete glucose series,

(Barford et. al.; 1971:Bessell et. al.; 1971: Foster et. al.; 1967:Pacak et. al.; 1969: Micheel and Klemer; 1961), fluoropentoses (Codington et. al.; 1966: Wright and Taylor; 1967: Wright et. al.; 1969: Wright and Fox; 1970), various other fluorinated hexoses (Adamson et. al.;1971: Taylor et. al.; 1972b: Kent et. al.; 1960) and glycosyl fluorides generally (Micheel and Klemer; 1961). Extensive studies have been reported in which fluorinated sugars of these types have been incorporated into a range of metabolic systems (Taylor; 1972), and their interactions with isolated enzymes have also been studied (Thomas et. al.; 1974). Furthermore, comparison of the properties of deoxyfluoro analogues with those of the corresponding hydroxy compounds have been made in studies of membrane transport (Barnett; 1972: Riley and Taylor; 1972). In only a few cases have systematic comparisons shown that deoxyfluoro compounds act as a hydroxy analogue. In most cases the apparent activity of a fluoro substituent as a hydroxy group can be explained in terms of the profound effects fluorine is known to have upon neighbouring groups.

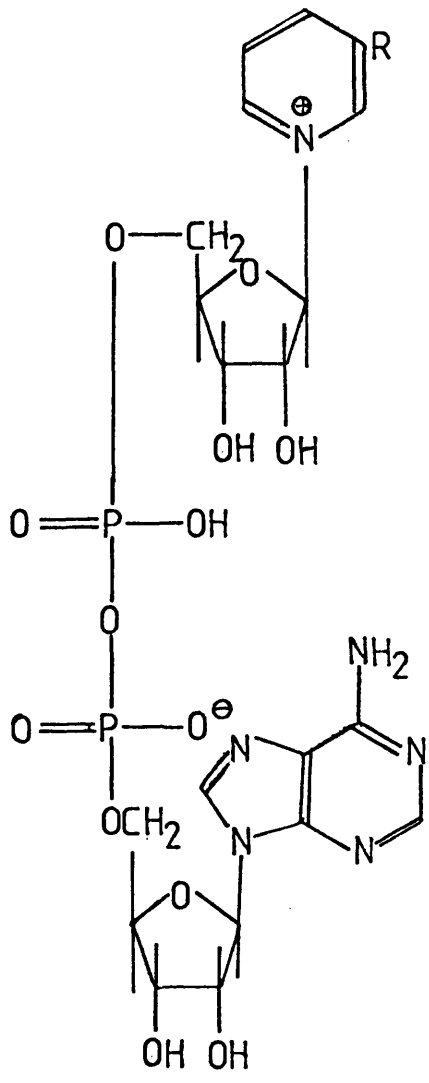
In view of the central role of NAD^+ in enzymically catalysed oxidation/reduction reactions it was thought that a fluorinated analogue of this coenzyme could be of great use as a probe, particularly by using ^{19}F n.m.r. spectroscopy.

SYNTHETIC METHODS

There are several positions in the NAD^+ molecule (Fig 9, R = $CONH_2$) into which fluorine could be substituted. It is theoretically possible to introduce fluorine into either of the ribofuranosyl rings, as well as into the adenine or nicotinamide rings. However, the nicotinamide ring is possibly the most attractive site in view of its involvement in oxidation-reduction reactions of the coenzyme, and on this ring the substitution of a trifluoroacetyl grouping for the amide moiety seemed to offer potentially the most accessible and useful analogue.

With a view to the use of ^{19}F n.m.r. spectroscopy, the trifluoroacetyl group offers the advantage of three fluorines in an identical environment giving a single, high intensity ^{19}F n.m.r. signal. This is of particular importance

Fig. 9



Formula for NAD^+ and its analogues.

as the concentration of the analogue in enzyme systems would be low. Hence it was decided to prepare 3-trifluoroacetylpyridine adenine dinucleotide (Fig. 9, R = $COCF_3$).

INTRODUCTION OF FLUORINE

There are two basic methods for the synthesis of fluoro-analogues of macro molecules. Either a fluorine is introduced directly into the macro molecule or a small part of the macromolecule is synthesised and then incorporated.

Bergstrom et.al. (1963) synthesised 9α-fluoro- 17β, 21-dihydroxy-4-pregnene-3, 20-dion-21-acetate by treatment of the 9, 11-olefinic derivative with 74% HF in pyridine at 2°C, although in poor yield (Fig. 10a).

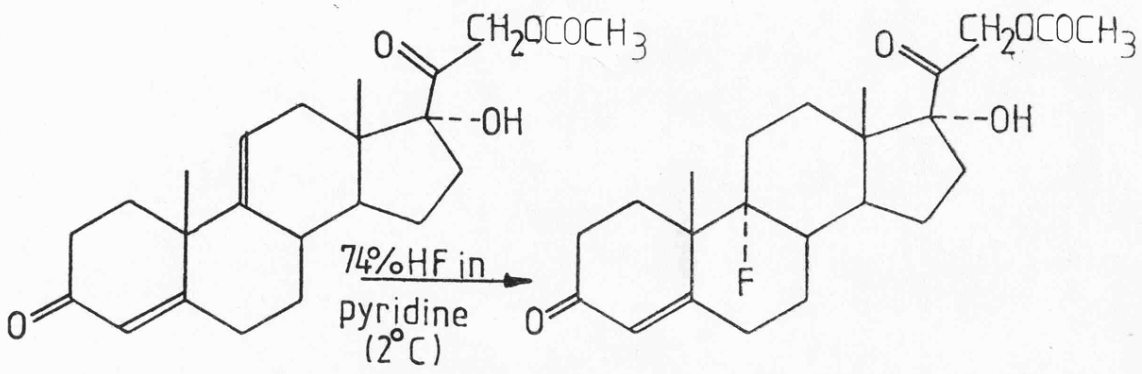
This reaction exemplified the addition of HF to a double bond.

Hirschmann et. al. (1956) made 9 α-fluoro-^{11} β-hydroxypregnenes, in high yield by opening an anhydro ring with anhydrous HF in tetra-hydrofuran and chloroform (Fig. 10b). Similar compounds have been synthesised (Pike et. al.; 1963) which have a pronounced anti-inflammatory activity.

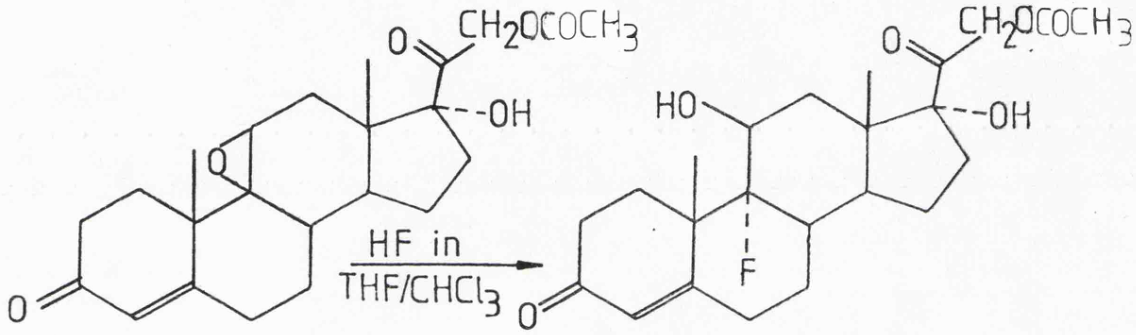
Codington et. al. (1961) demonstrated the opening of a five-membered oxygen ring with hydrogen fluoride when they synthesised 2'-deoxy-2'-fluorouridine by the action of HF in dioxan on 2, 2'-anhydro-1-(β-D-arabinofuranosyl) uracil (Fig. 10c).

Halides or sulphonate esters can be replaced by fluorine by the action of a metal fluoride. Potassium fluoride has, in the past, been the metal fluoride most widely used as a fluorinating agent, although the fluorides of caesium and rhubidium are often more active in this respect. Sodium fluoride has a low solubility, and lithium fluoride is a poor nucleophile because of its strongly ionic crystal lattice and large hydration shell.

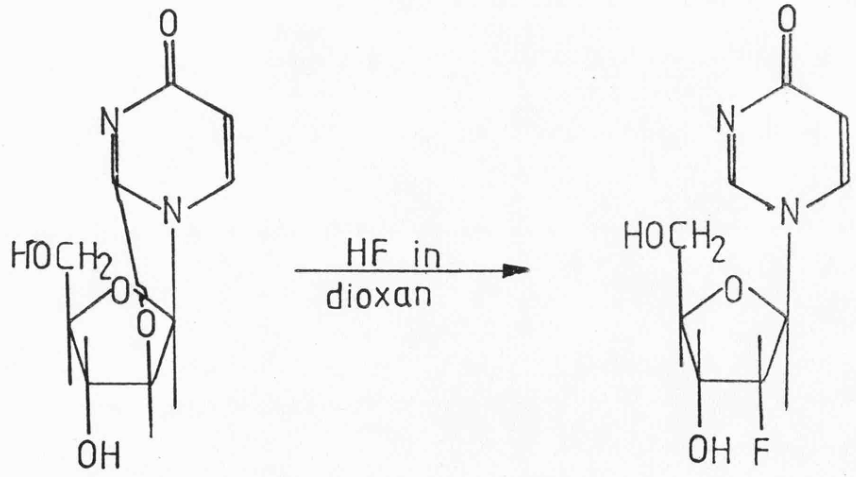
Fig. 10.



(a) Preparation of 9 α-fluoro-17 β,21-dihydroxy-4-pregnene-3,20-dion-21-acetate.



(b) Preparation of 9 α-fluoro-11 β-hydroxypregnenes.



(c) Preparation of 2'-deoxy-2'-fluorouridine.

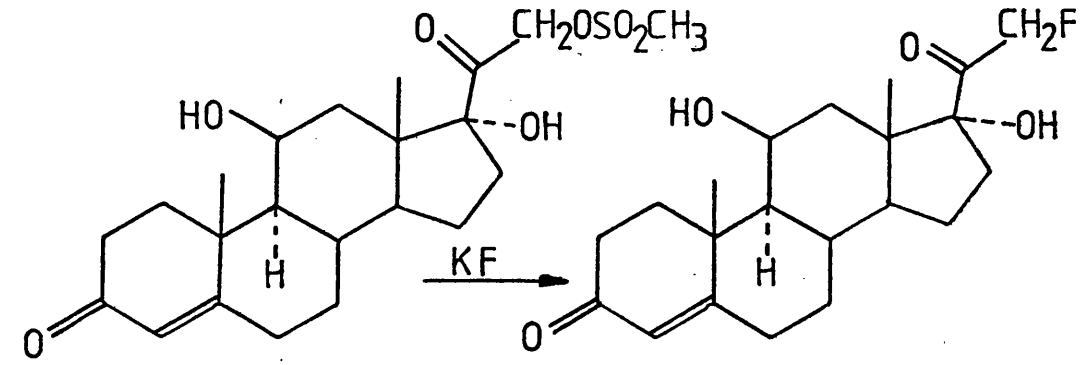
Fluorinations involving HF.

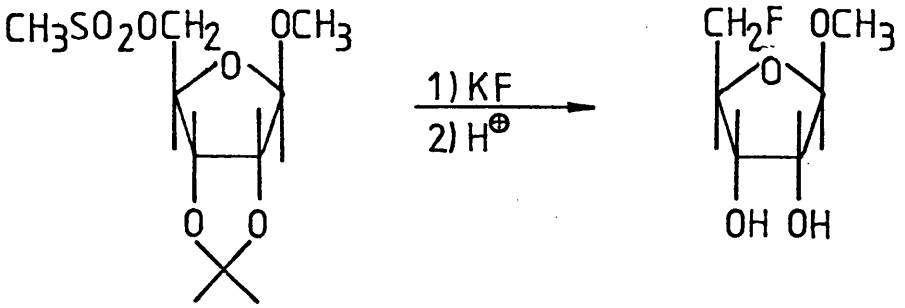
Potassium fluoride can be used to displace other halogens from acyl halides, α-haloesters, amides, nitriles and primary alkyl halides in polar solvents. Dry potassium fluoride in polar solvents has also been used to replace oxygen-bonded groups, particularly p-toluene sulphonyl and methanesulphonyl esters, which are excellent leaving groups, easily replaced when in a primary position. This is a mild method of replacement, particularly valuable for molecules containing sensitive groups, and allows for the conversion of an alcohol to a fluoride *via* a sulphonate ester (Fig. 11).

Application of fluoride exchange reaction using p-toluene sulphonate within ring systems had proven difficult until Henbest and Jackson (1962) described the action of tetra-n-butyl ammonium fluoride in acetone or butanone on C-3 and C-17 p-toluenesulphonyl substituted steroids. The reactions proceeded with very high yield and inversion of configuration, and thus these workers were able to demonstrate useful routes to 3α- and 3β-fluorocholestane (Fig. 12a) and 17α-fluorotestosterone (Fig 12b). The suitability of tetraalkyl ammonium fluorides as fluorinating agents is reflected in the fact that they are highly dissociated and extremely soluble in organic solvents. With the aid of suitable blocking groups, this reaction was adopted for use in the synthesis of secondary fluorocarbohydrates. Foster *et. al.* (1967) converted 1, 2; 5, 6-di-O-isopropylidene-3-O-p-toluenesulphonyl-D-allofuranose to 3-deoxy-3-fluoro-1,2; 5,6-di-O-isopropylidene-α-D-glucofuranose in 74% yield using tetra-n-butylammonium fluoride in acetonitrile. 3-Deoxy-3-fluoro-L-idose (Brimacombe *et. al.*; 1970) and 4-deoxy-4-fluoro-D-galactose (Marcus and Westwood; 1971) have since been synthesised by similar routes.

When a smaller molecule is fluorinated, and then incorporated into the macromolecule any of the methods used for the direct fluorination of macromolecules may be used in the fluorination of the smaller molecules

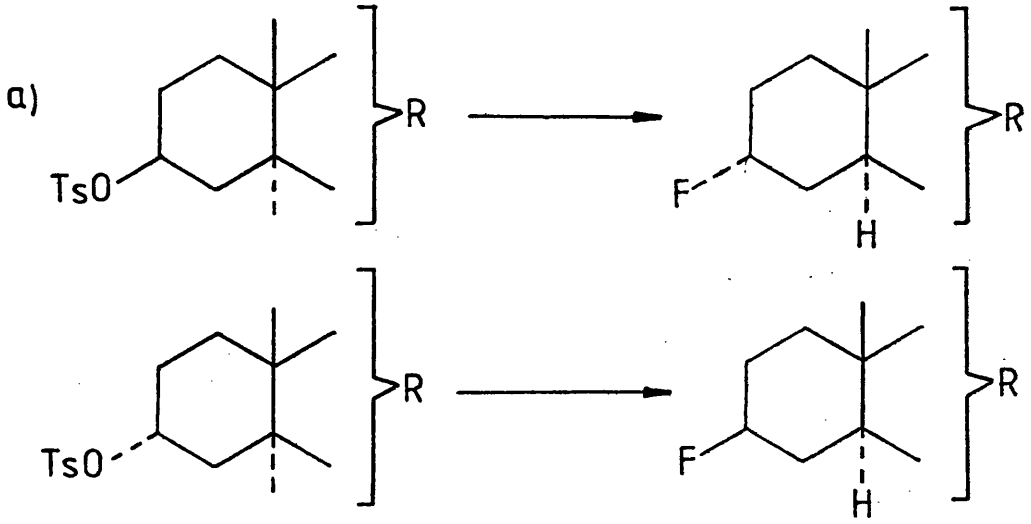
Fig. 11.



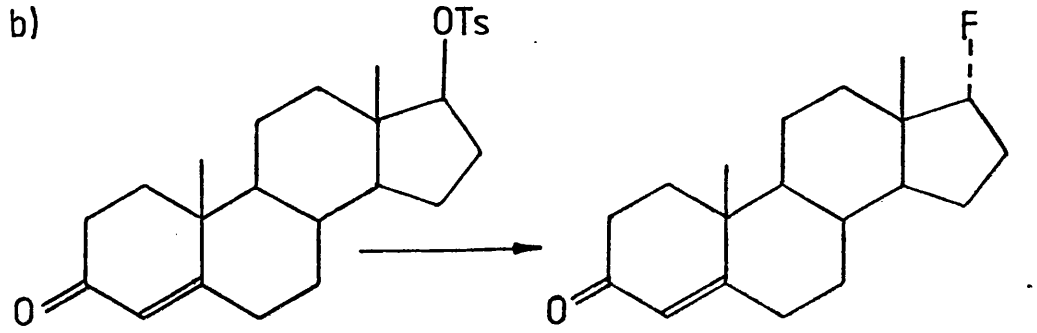


Fluorination using a metal fluoride and a leaving group.

Fig. 12.



R= Remainder of steroid molecule.



Ts = p-Toluenesulphonyl group

Fluorinations using tetra-n-butyl ammonium fluoride.

(Fig. 13).

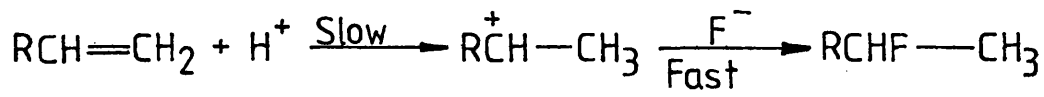
One other method of fluorination that is of particular interest in the field of the synthesis of biologically active molecules is the replacement of hydrogen in a reactive methylene group using perchloryl fluoride. Inman et. al. (1958) have described the fluorination of diethyl malonate in ethanol to yield diethyl difluoromalonate in 84% yield. However, reinvestigation of this method by Gershon et. al. (1966) has shown that a mixture of five components is present, diethyldifluoromalonate only accounting for 36% of the theoretical yield. Gershon et. al. (1967) also investigated the action of perchloryl fluoride on substituted ethyl cyanoacetate and studied the toxicity of the products.

The general method for the introduction of fluorine into a compound with an active methylene group using perchloryl fluoride is described below. The compound to be fluorinated is stirred in an inert solvent under nitrogen, a base added, and perchloryl fluoride bubbled through the cooled solution. The range of compounds which has been made by this method is great (Fig. 14) but that prepared by Inman et. al. (1958) is of particular interest from a biochemical point of view. They prepared diethyl difluorooxaloacetate, a potential precursor for difluorooxaloacetate, itself a useful probe for the active site of malate dehydrogenase (Smith et. al.;1977) and aspartate transaminase (Briley et. al.; 1977).

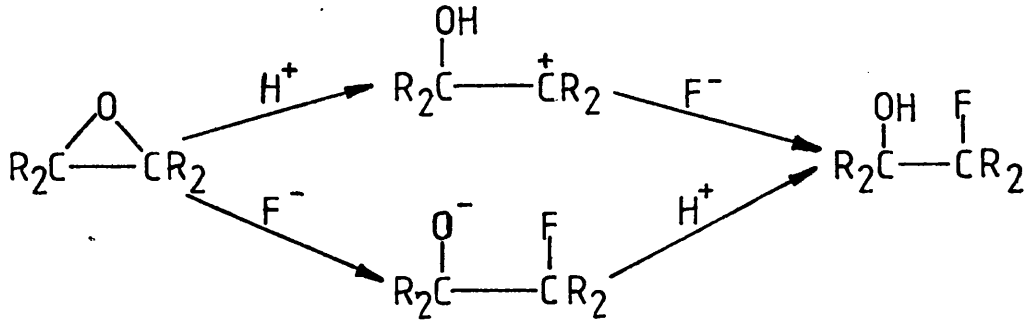
There have been many mechanisms proposed for the action of perchloryl fluoride as an active methylene group, but that proposed by Sheppard (1969) fits the observed facts. He proposed, as a simple mechanism for the reaction of anions with perchloryl fluoride, that the most nucleophilic centre in the anion always attacks the chlorine, and never the fluorine, which is more electronegative . With localised nucleophiles, such as alkoxides, fluoride ion is displaced, but with mesomeric ions an intramolecular, cyclic transfer of fluoride ion can occur in the intermediate to form a carbon-fluorine bond. The strong driving force

Fig. 13.

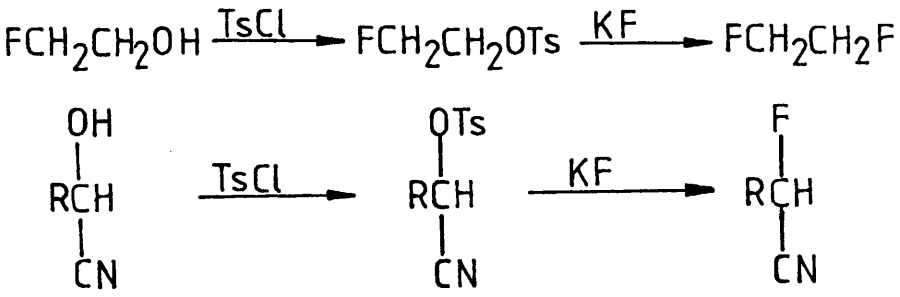
(a) Addition of HF to an alkene.



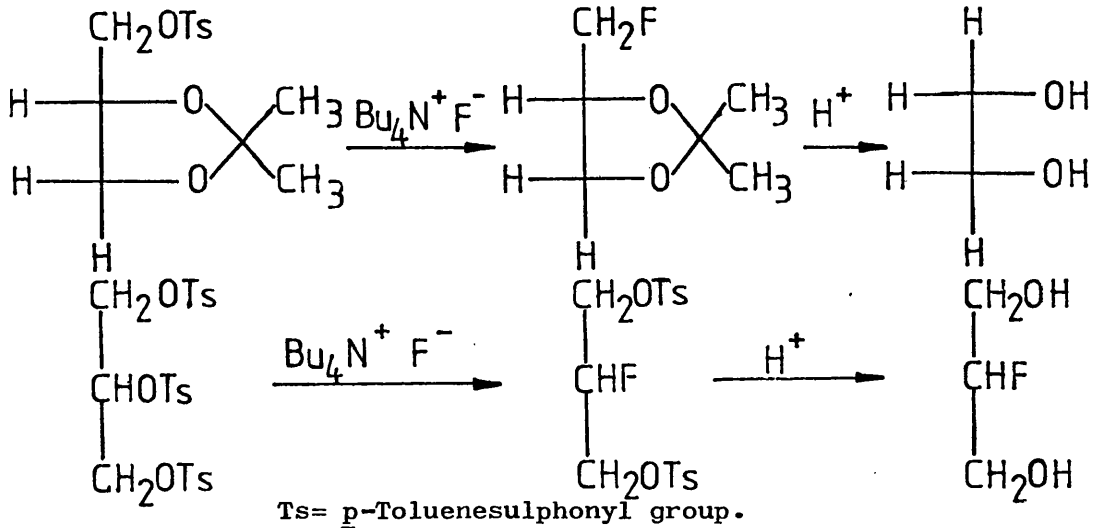
(b) Addition of XF to an anhydro ring.



(c) Displacement of sulphonate esters by metal fluorides.



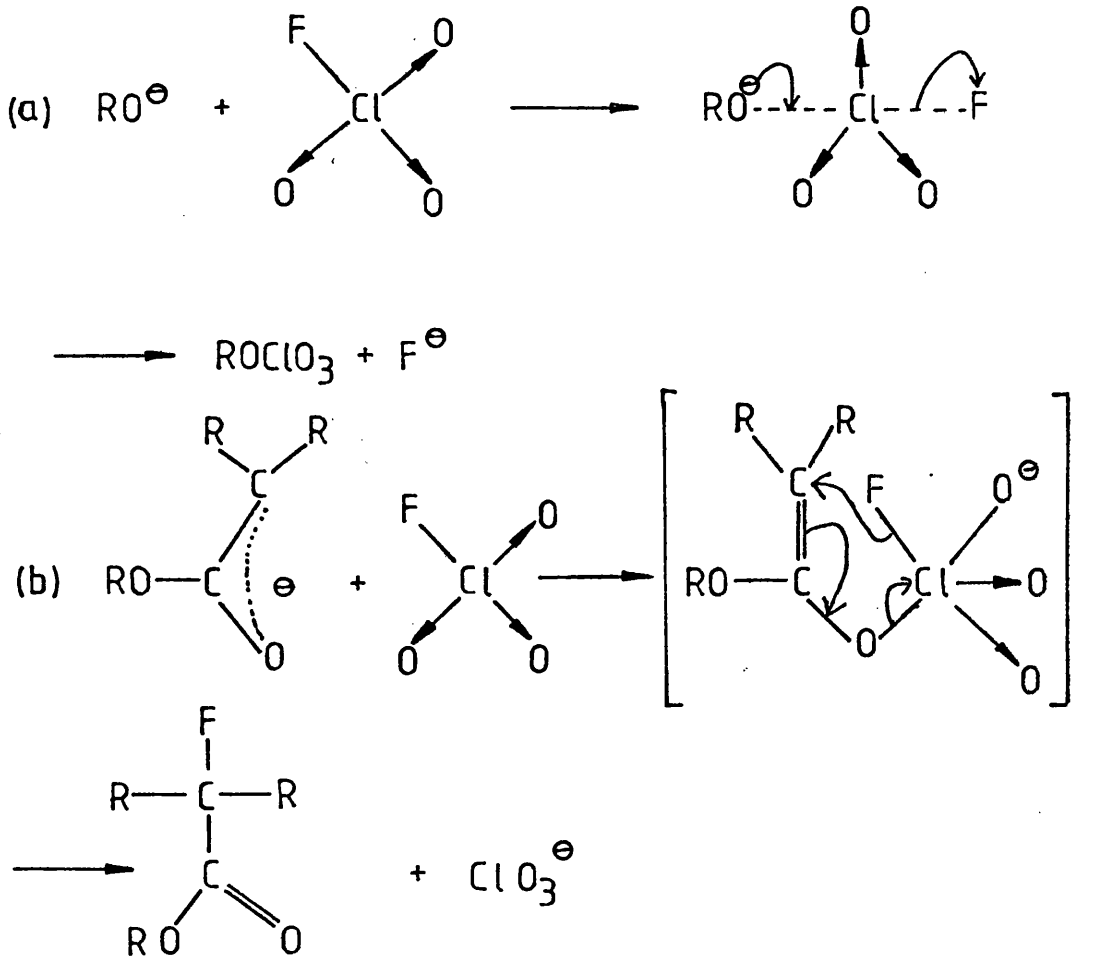
(d) Displacement of sulphonate esters by tetraalkylammonium fluoride.



Ts= p-Toluenesulphonyl group.

Incorporation of fluorine into smaller molecules.

Fig. 14.



Ø = Phenyl group.

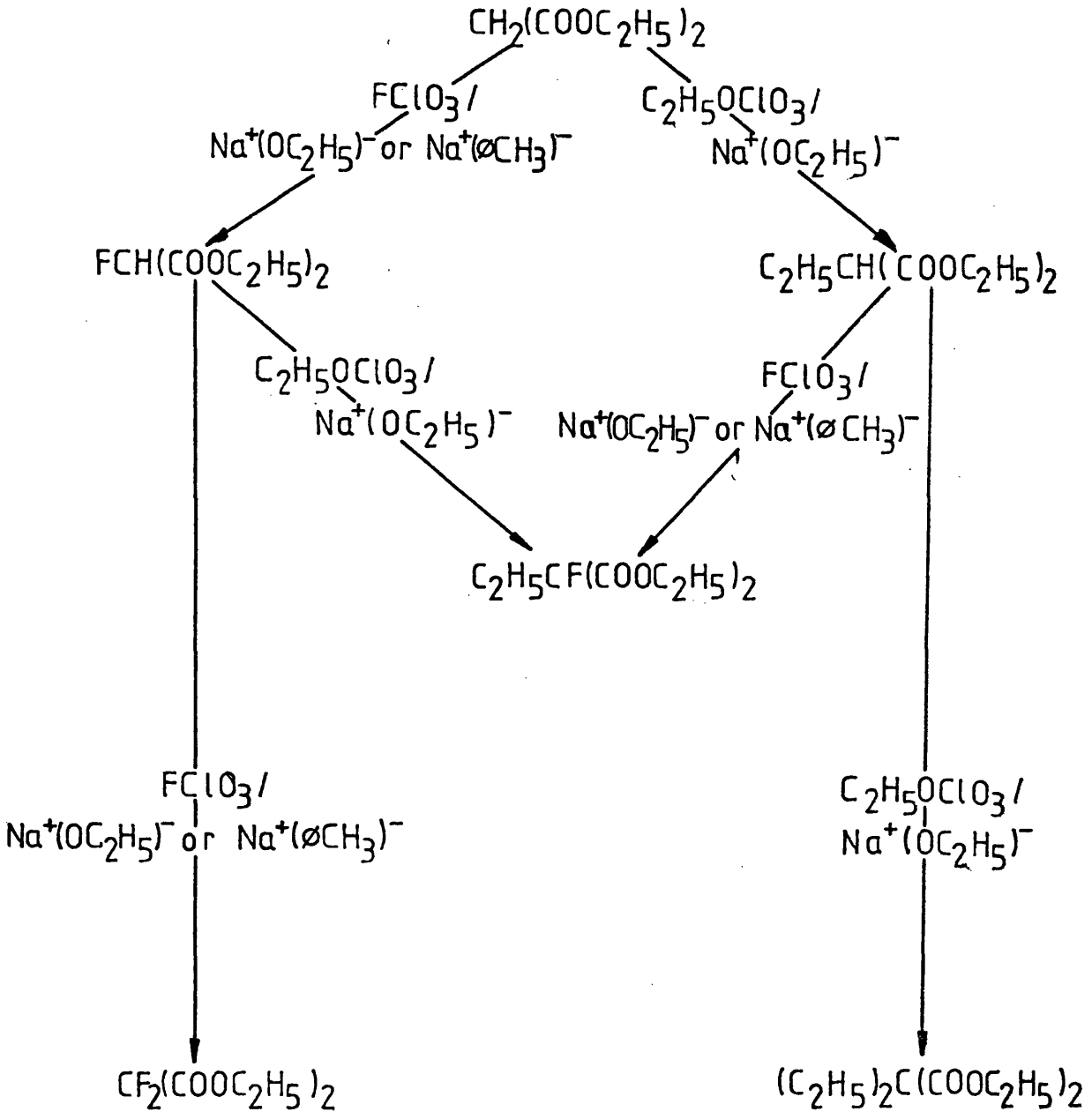
Mechanism of fluorination with perchloryl fluoride.

for this reaction is the high energy gained by the formation of a carbon-fluorine bond, and the fact that fluorine never has to achieve that highly unfavourable energy state in which it has a positive charge. This mechanism also explains the reaction of perchloryl fluoride with organometallic reagents, such as that described by Schlosser et. al. (1969) (Fig. 14c). It explains the fact that phenyllithium gives perchloryl benzene rather that fluorobenzene, whereas 2-lithiothiophene gives 2-fluorothiophene in high yield (Schuetz et. al.; 1963). The mechanism also forecasts the formation of by-products such as ethyl perchlorate when the reactions are performed in ethanol, as was described by Gershon et. al. (1966) (Fig. 15). As can be seen ethyl perchlorate is capable of reacting with both starting material and intermediates and the final reaction mixture contains numerous by-products, as well as the desired compound. Hence it can be seen that the choice of solvent for perchloryl fluoride reactions is crucial. Such difficulties may be overcome by using a solvent system of toluene and dioxan (3:1) which was, for example, used by Briley et. al. (1977_a) in their preparation of diethyl difluorooxaloacetate.

Synthetic modifications to pyridine compounds

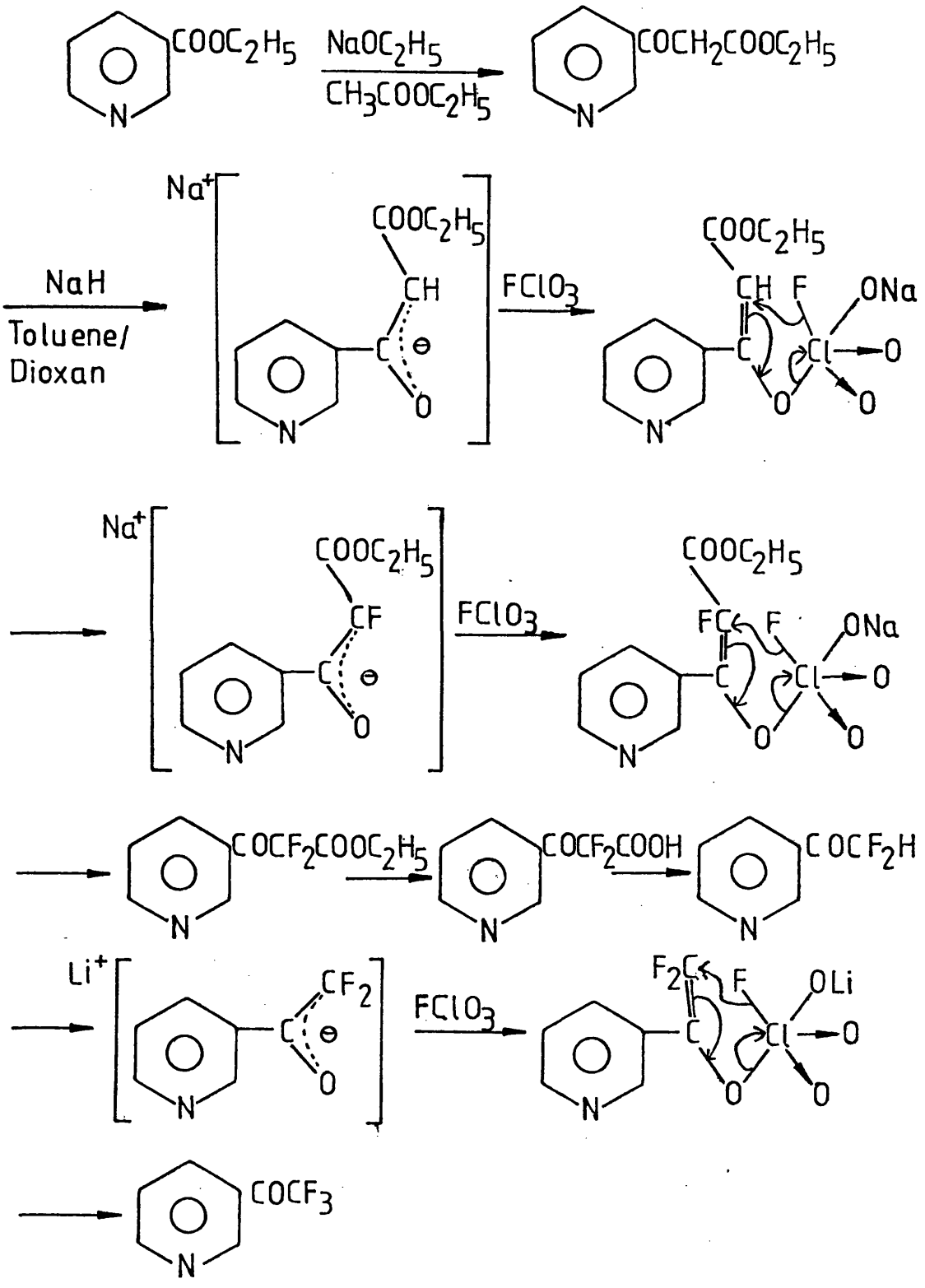
In view of the proposed mechanism for fluorination reactions involving perchloryl fluoride, and the expertise gained at Bath University in the use of this reagent, it was decided to attempt the preparation of trifluoroacetylPyridine from ethyl nicotinoylacetate using perchloryl fluoride as the fluorinating agent. There are many methods published for the preparation of ethyl nicotinoylacetate (Koelsch; 1945), and a reaction sequence was accordingly proposed using this as the starting material (Fig. 16). As can be seen the proposed reaction sequence is compatible with the mechanism suggested by Sheppard, and the reactions

Fig. 15.



By-products obtained when ethanol is used as a solvent in fluorinations involving perchloryl fluoride.

Fig. 16.



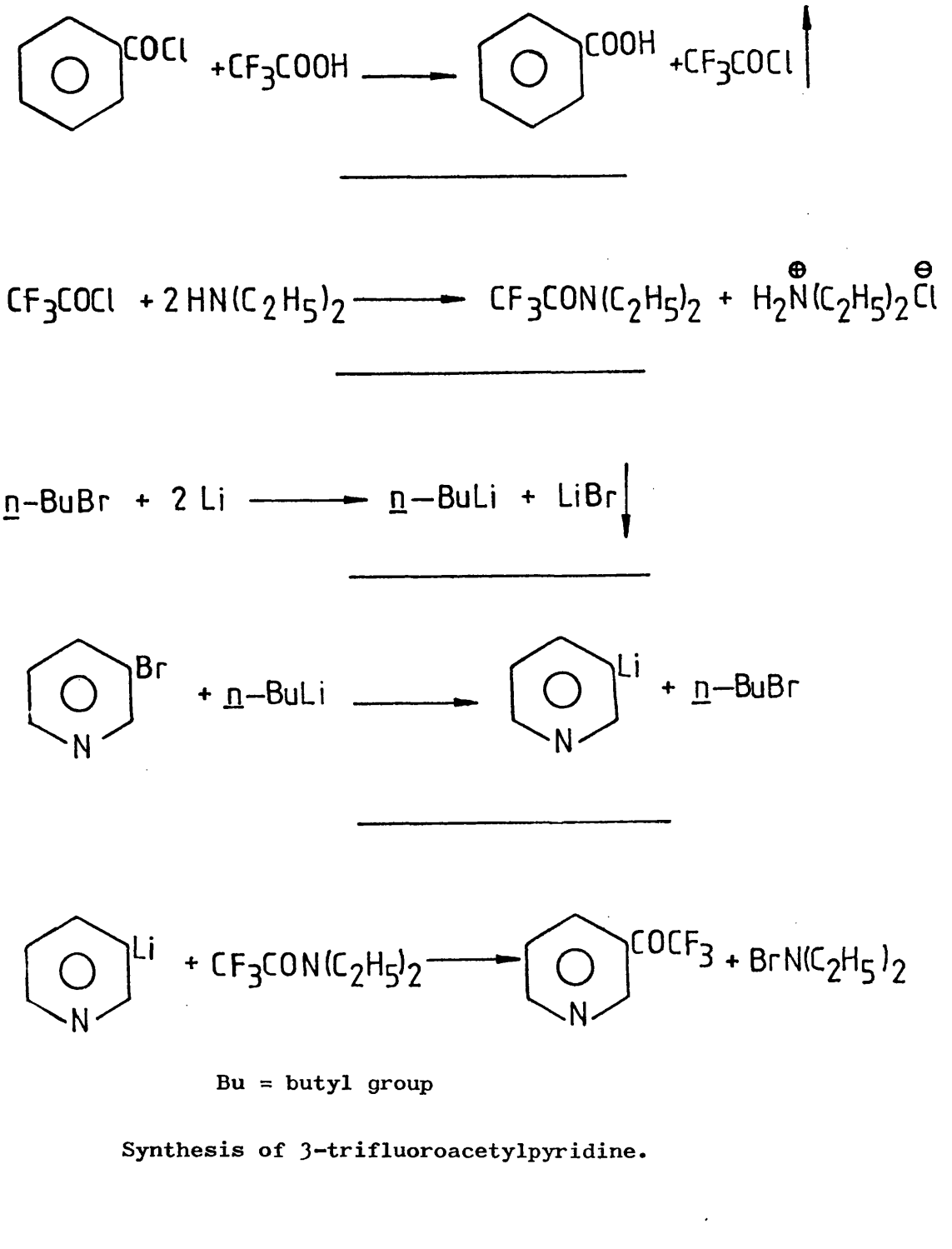
Synthesis of 3-trifluoroacetylpyridine using perchloryl fluoride.

other than the fluorination steps, are well documented.

Salvador and Saucier (1971) developed a method for the preparation of a series of trifluoroacetylpyridines from the corresponding lithiopyridine derivative. They based their method on that of Zaitseva et. al. (1961), and it involves the reaction of N, N-diethyltrifluoroacetamide with the appropriate lithiopyridine derivative (Fig. 17). Information was also given by Salvador and Saucier concerning the spectroscopic data of these compounds, which became hydrated to form some of the few stable gem-diols which have been isolated in the pure state. The gem-diol of thenoyltrifluoroacetone (Fig. 18a) was one of the few compounds of this type which had previously been isolated. Its stability is attributed to intramolecular hydrogen bonding.

Similarly, the four gem-diols reported by Salvador et. al. (Fig. 18b) are also stablised by hydrogen bonding. Their remarkable stability is exemplified by a number of properties. The 2- and 4- pyridyl gem diols are, for example, recrystallisable from anhydrous organic solvents, while the 4-pyridyl compound can be sublimed at 120°C without loss of water. This stability can be accounted for by the inductive effects of the trifluoromethyl group on the acidity of the hydroxyl groups. Middleton and Lindsey (1964) showed that fluoroalcohols form extremely strong hydrogen bonds (ca. 6 Kcal/mole) with electron rich atoms. This enables them to form stable 1:1 complexes with a series of bases. The stability of these complexes is a function of the acidity of the alcohols and the basicity of the bases involved. Like the fluoroalcohols, the hydroxyl function of some trifluoromethyl ketone gem-diols have been estimated (Stewart and Van der Linden; 1960) to be quite acidic, and they should thus form strong bonds with basic atoms.

Fig. 17.

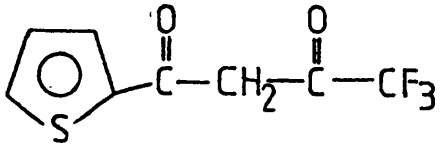


Bu = butyl group

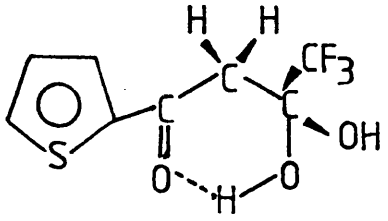
Synthesis of 3-trifluoroacetylpyridine.

Fig. 18.

(a) i Thenoyltrifluoroacetone.

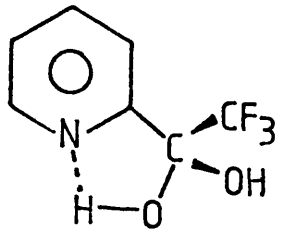


ii Thenoyltrifluoroacetone gem-diol.

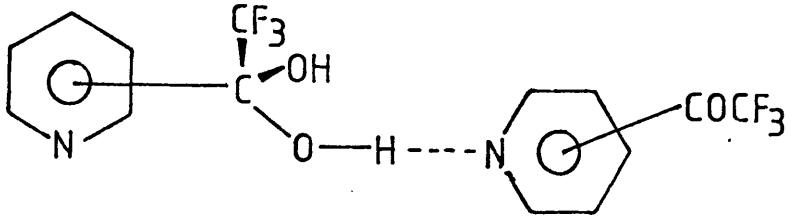


(b) Trifluoroacetylpyridines.

i Intra-molecular.



ii Inter-molecular.



Stabilising effect of hydrogen bonding in gem - diols.

The pyridyl gem-diols all contain basic nitrogen atoms and hence would be susceptible to both intra- and inter-molecular hydrogen bonding. As expected, the 3-pyridyl compound is less stable than either the 2- or 4-pyridyl compounds because of the mesomeric effects of the pyridine ring.

^{1}H N.m.r. spectroscopy showed a singlet for the hydroxyl groups in each of the pyridyl compounds, ruling out the possibility of strong, intramolecular hydrogen bonding, leaving intermolecular hydrogen bonding as the main stabilising influence.

3-Lithiopyridine , the starting material for the synthesis of 3-trifluoroacetylpyridine , may be prepared by the method of Wibaut et. al. (1951).

Nucleoside synthesis

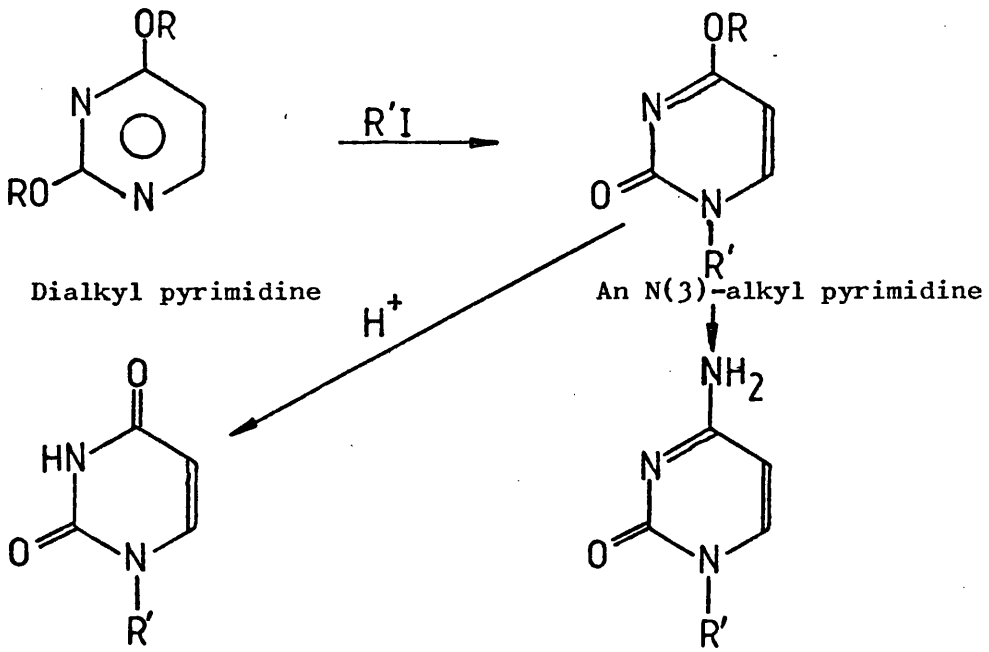
Nucleotides and their analogues have been synthesised by both chemical and enzymic methods, the first stage in a chemical synthesis being the preparation of a nucleoside.

A natural nucleoside was first synthesised by Todd et. al. (1947) using the method of Hilbert and Johnson (1930) who had found that a dialkoxy pyrimidine reacts at room temperature with one mole of an alkyl halide to give N(3)-alkylpyrimidine (Fig. 19a). (i.e. the new alkyl group is bound to the same nitrogen as that to which the sugar is attached in a nucleoside) On treatment with acid the pyrimidine is converted to an uracil, and with alcoholic ammonia the corresponding cytosine is formed.

With acetobromoglucose, a halo sugar, in place of an alkyl halide, a completely analogous series of reactions takes place, and the β-D-glucopyranosides of uracil and cytosine were obtained by Hilbert and and his co-workers (1930a; 1936). In the synthesis of cytidine (Todd et. al.; 1947) the reactants were diethoxypyrimidine and triacetylribofuranosyl bromide, deacylation of the product with

Fig. 19.

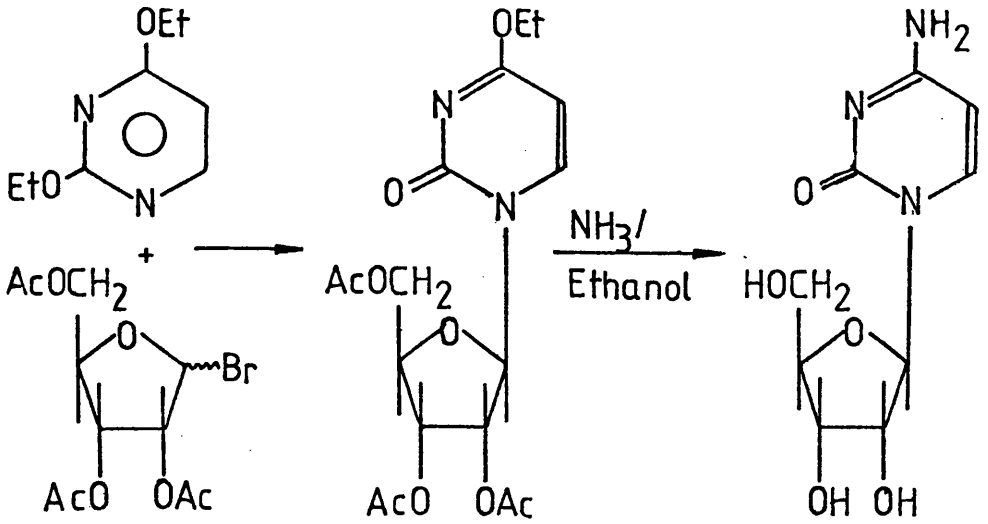
(a) Reaction of pyrimidines with alkyl halides.



An uracil

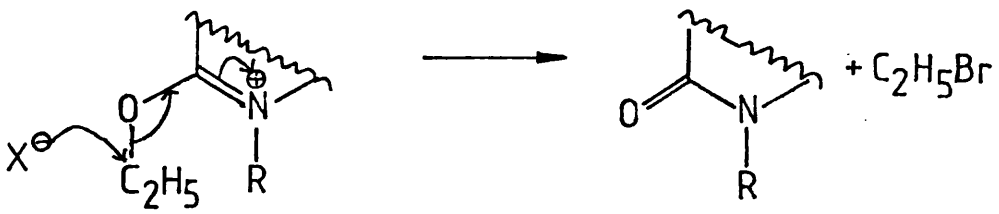
A cytosine

(b) Synthesis of cytidine.



Ac = acetyl group; Et = ethyl group.

(c) Mechanism of the reaction.



alcoholic ammonia giving cytidine (Fig. 19b).

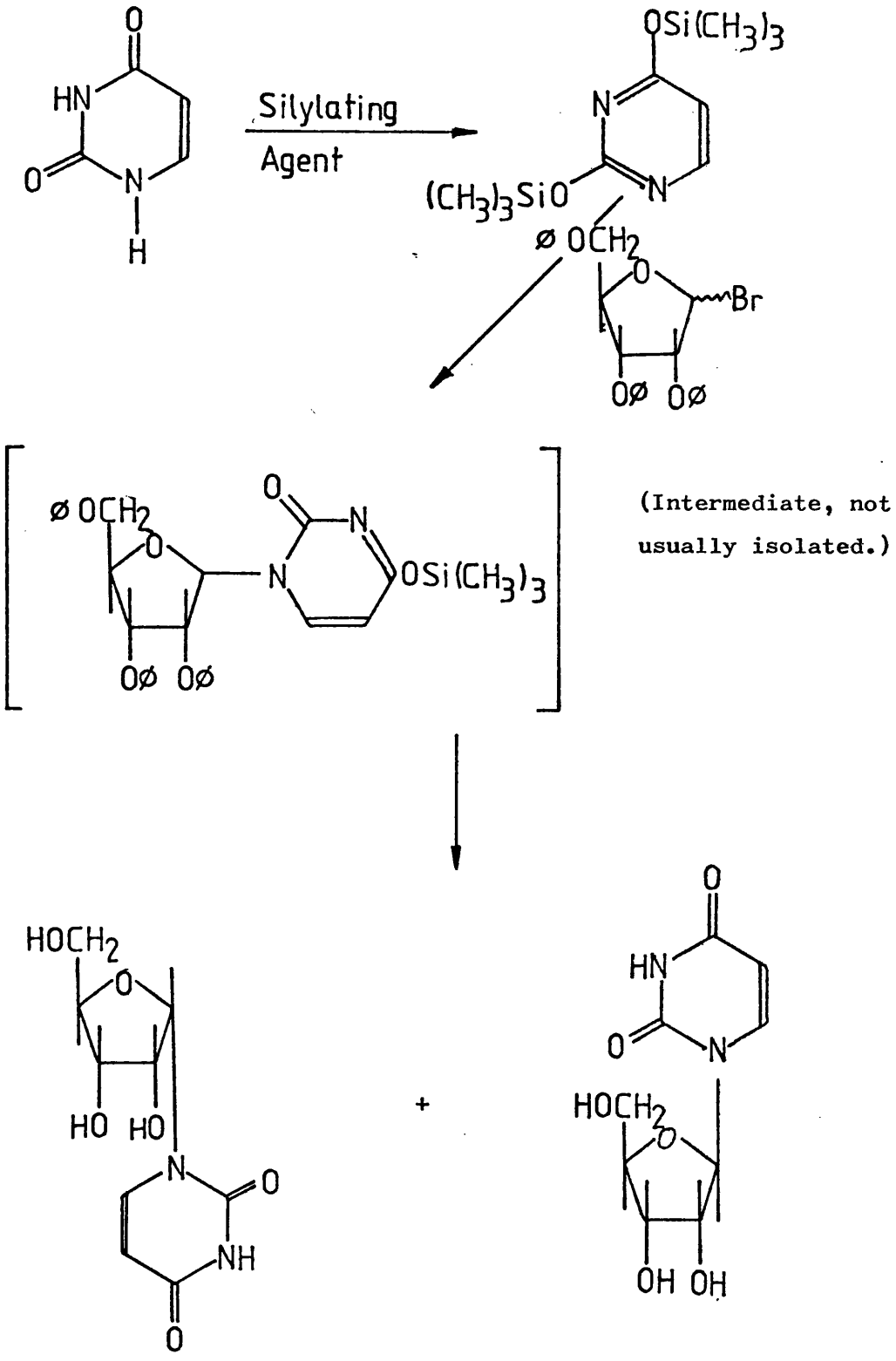
The mechanism of the reactions of dialkoxypyrimidines with halogeno compounds, and with acids is fundamentally the same. The first step is the addition of a proton, alkyl or glycosyl group to the nitrogen, followed by attack by the halide ion on the alkoxy grouping (Fig. 19c).

A modification of this method, developed by Iwia et. al. (1964), has distinct advantages over the original method; the principal one being that silylated derivatives may be prepared in excellent yields directly from pyrimidines such as cytosine, uracil or thymine. Silylation may be readily accomplished with either chlorotrimethylsilane, hexamethyldisilazane or trimethylsilyl-N-(trimethylsilyl) acetamidate, the latter of which is reported to be a very powerful silylating agent.

As compared with the Hilbert-Johnson synthesis, in which alkoxy derivatives are employed, silylated pyrimidines react sluggishly with O-acylglycosyl halides, and more rigourous conditions, such as heating the reactants together at high temperature, are required. The residual trimethylsilyl groups thus formed are readily cleaved by water or simple alcohols, and the completely protected nucleoside is never isolated as such. Instead, they are usually dissolved in either methanol or ethanol which affords the acylated derivatives directly. Thus the reaction step in the Hilbert-Johnson synthesis, in which a residual alkoxy group of the glycosylated pyrimidine is separately cleaved has been eliminated.

The trimethylsilyl procedure frequently affords a mixture of anomeric nucleosides (Fig. 20) (the Hilbert-Johnson synthesis gives predominently the β-isomers) and is also broader in its field of application.

Fig. 20.



1 α-D-ribofuranosyluracil. Uridine

Ø = benzoyl group.

Synthesis of pyrimidine nucleosides using silylated derivatives.

The method has been extended to the preparation of uracil nucleosides containing D-arabinofuranose or D-lyxofuranose residues, thymine nucleosides containing D-arabinofuranose, D-lyxofuranose or D-ribofuranose residues, and an adenine and a hypoxanthine nucleoside, both containing a D-glucopyranose residue (Nishimura et. al.; 1964a and b: 1965: 1968).

Fusion of an O-acylglycoside halide with a silylated purine or pyrimidine at high temperature (ca. 190°C) as in the previous synthetic routes is destructive to the halides involved. However, by using more reactive per-O-acyl-2-deoxyglycosyl halides milder conditions may be used. Durr et. al. (1967) fused 2-deoxy-3, 4, 6-tris-O-(4-nitrobenzoyl)-α-D-arabinohexosyl bromide with 3,5-bis-(trimethylsiloxy)-as-triazine (prepared by the silylation of 3,5-dioxo-2, 3, 4, 5-tetrahydro-as-triazine with hexamethylsilazane) at 85°C to give the acylated derivative in 35% yield. Deacylation yielded 3, 5-dioxo-2-(2′-deoxy-β-D-arabino-hexopyranosyl)-2,3,4,5-tetrahydro-as-triazine (1-(2′-deoxy-β-D-glycopyranosyl)-6-azauracil). The synthesis is not complicated by the formation of the undesired N-4 isomer, as was the case when the alkoxy derivatives were used. In a similar experiment Shen and his co-workers (1968) synthesised 3,5-dioxo-2-(2′-deoxy-β-D-erythropentafuranosyl)-6-trifluoromethyl-2,3,4,5-tetra-hydro-as-triazine (2'-deoxy-5-trifluoromethyl-6-azauridine) by reacting 6-trifluoromethyl-3,5-bis (trimethylsiloxy)-as-triazine with 2-deoxy-3,5-bis-O-(4-nitrobenzoyl)-D-erythropentosyl chloride at 150°C for 30 minutes.

A significant improvement in the trimethylsilyl method was made by Wittenberg et. al. (1968) who eliminated the necessity for high temperatures in the condensation, by dissolving an O-acylglycosyl

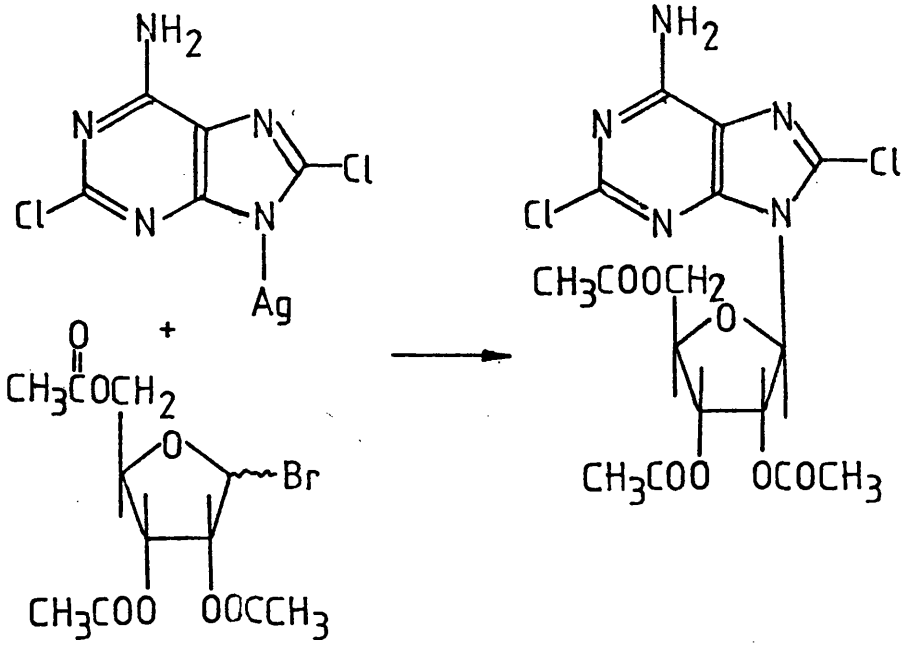
halide and a trimethylsilylated pyrimidine in a solvent, such as benzene, and subsequently adding silver perchlorate. The reaction is very exothermic and appears to proceed *via* a highly reactive O-acylglycosyl perchlorate (Birkhofer *et. al.*; 1964). The condensation is also facilitated by the addition of mercuric oxide and mercuric bromide to the reaction mixture, although in this case heating is required before the reaction will proceed. Alternatively, silver perchlorate and mercuric oxide/mercuric bromide may be used to prepare acylated thymine nucleosides derived from β-D-galactopyranose, α-L-arabinopyranose and β-D-ribofuranose in 45 to 48% yield. Thymine nucleosides containing 2-deoxy-D-*erythro*-pentafuranose, 2-deoxy-D-*erythro*-pentapyranose or 2-deoxy-D-*threo*-pentapyranose residues and 1-(2'-deoxy-D-*erythro*-pentopyranosyl)uracil have also been prepared by a similar method (Wittenberg; 1968: Wittenberg *et. al.*; 1968). Winkley and Robins (1968; 1969) used the trimethylsilyl method to prepare 2,4,6-trisubstituted pyrimidines which were difficult to prepare by other methods. The conditions of the condensation were modified by dissolving the trimethylsilylated pyrimidine and the O-acylglycosyl halide in dry acetonitrile, and allowing the reaction to proceed at room temperature for several days. Although time consuming, this variation of the method eliminates the necessity for either heating or using other reagents, and is undoubtedly the least destructive procedure, as shown by the high yields of acylated nucleosides. 5,6-Dimethyluridine, 6-methylcytidine and 1 β-D-ribofuranosyl-barbituric acid have been prepared by this method. However, with silylated derivatives of 6-aminouracil, 6-methyluracil and 6-(methylthio)-uracil glycosylation takes place at N-3 rather than N-1.

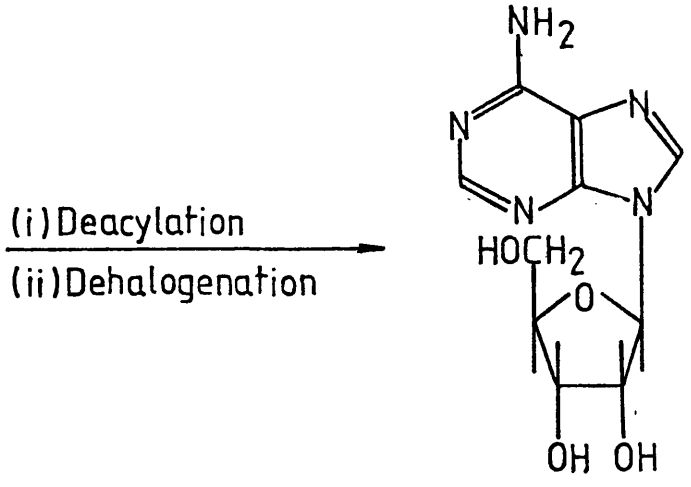
It was shown by Fischer and Helferich (1914) that purine nucleosides may be synthesised by the reaction of the silver salt of 2, 8-dichloroadenine with a halogeno-sugar. Todd et. al. (1948) reacted the silver salt of 2,8-dichloroadenine with 1-bromo-2,3,5-triacetylribofuranose, deacylated the product, then catalytically dehalogenated the nucleoside to yield adenosine (Fig. 21). The reason for using 2, 8-dichloroadenine is that adenine itself is sufficiently basic to dehydrohalogenate the sugar derivative.

Davoll (1948; 1951) found that it was simpler to acylate the amino group and use, for example, N-benzoyladenine. Further he showed that better yields were obtained using purine mercuric chloride rather than silver salts. The reaction, with these modifications, which still constitutes the best general method for synthesising purine nucleosides is illustrated by the synthesis of guanosine from the diacetylaminopurine mercuric chloride (Fig. 22).

The fusion method, developed by Sato and his co-workers (1961) is a procedure for the synthesis of purine nucleosides, and is simply the fusion of an acylated sugar with a purine in the presence of an acidic catalyst under reduced pressure. The method is applicable to purines, and further substitution of the purine to provide protecting groups for such functions as amino, oxo or thio, which might already be present, is not necessary. The use of per-O-acylglucose is of distinct advantage, eliminating the necessity for transforming it into the corresponding, relatively unstable O-acyl glycosyl halide, hitherto the main intermediate used in the synthesis of purine nucleosides. A wide variety of purine nucleosides have been prepared by this method, in yields of up to 80% (Sato; 1968: Sato et. al.; 1965: Pan et. al.; 1967: Montgomery et. al.; 1968.)

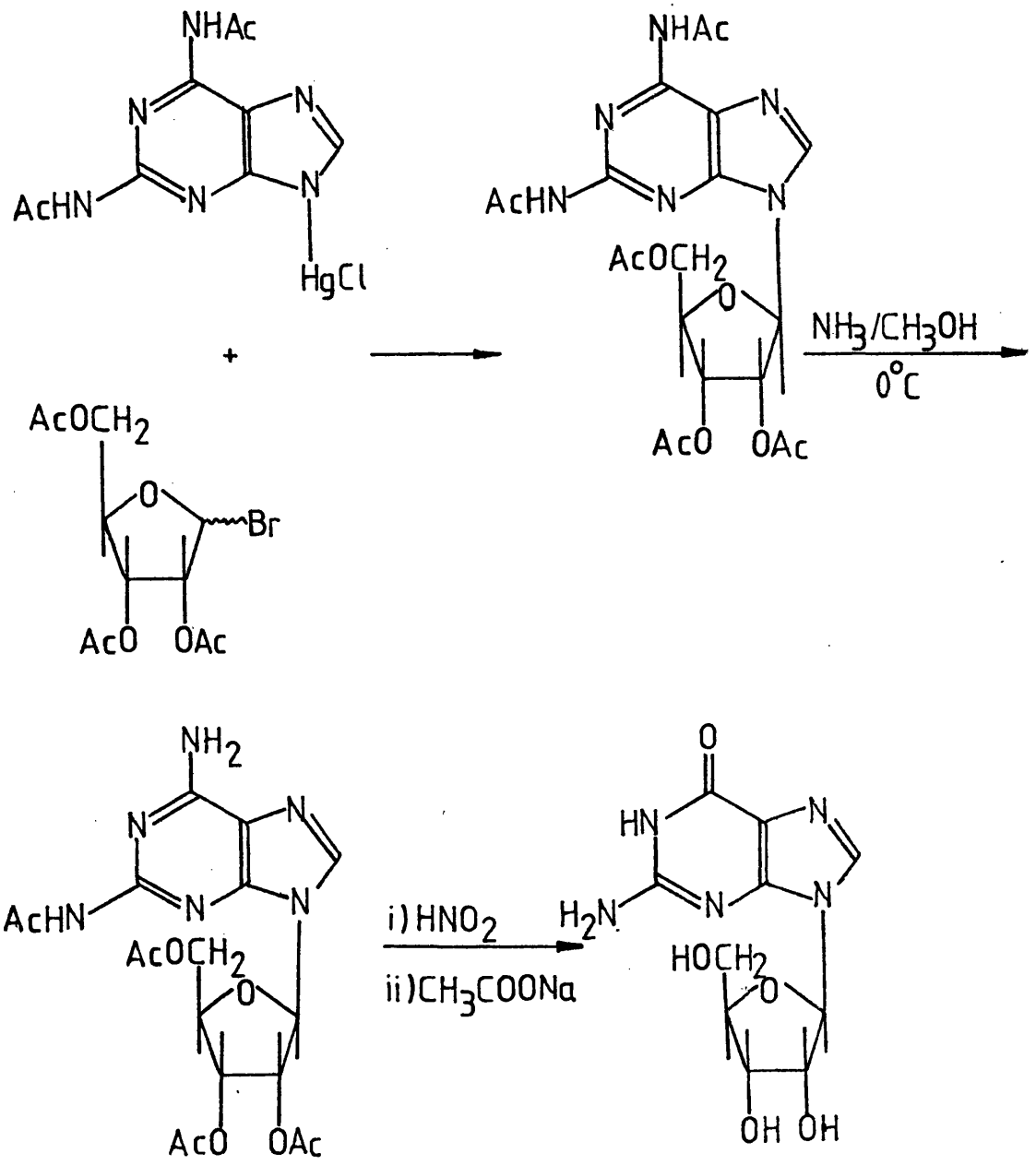
Fig. 21.





Synthesis of adenosine.

Fig. 22.



Ac = acetyl group

Synthesis of guanosine.

The synthesis of 2-bromoadenosine (Montgomery and Hewson; 1964: 1968) illustrates the procedure, and serves to show how a substituted purine may lead to a nucleoside that is readily transformed into another nucleoside with the same sugar residue (Fig. 23).

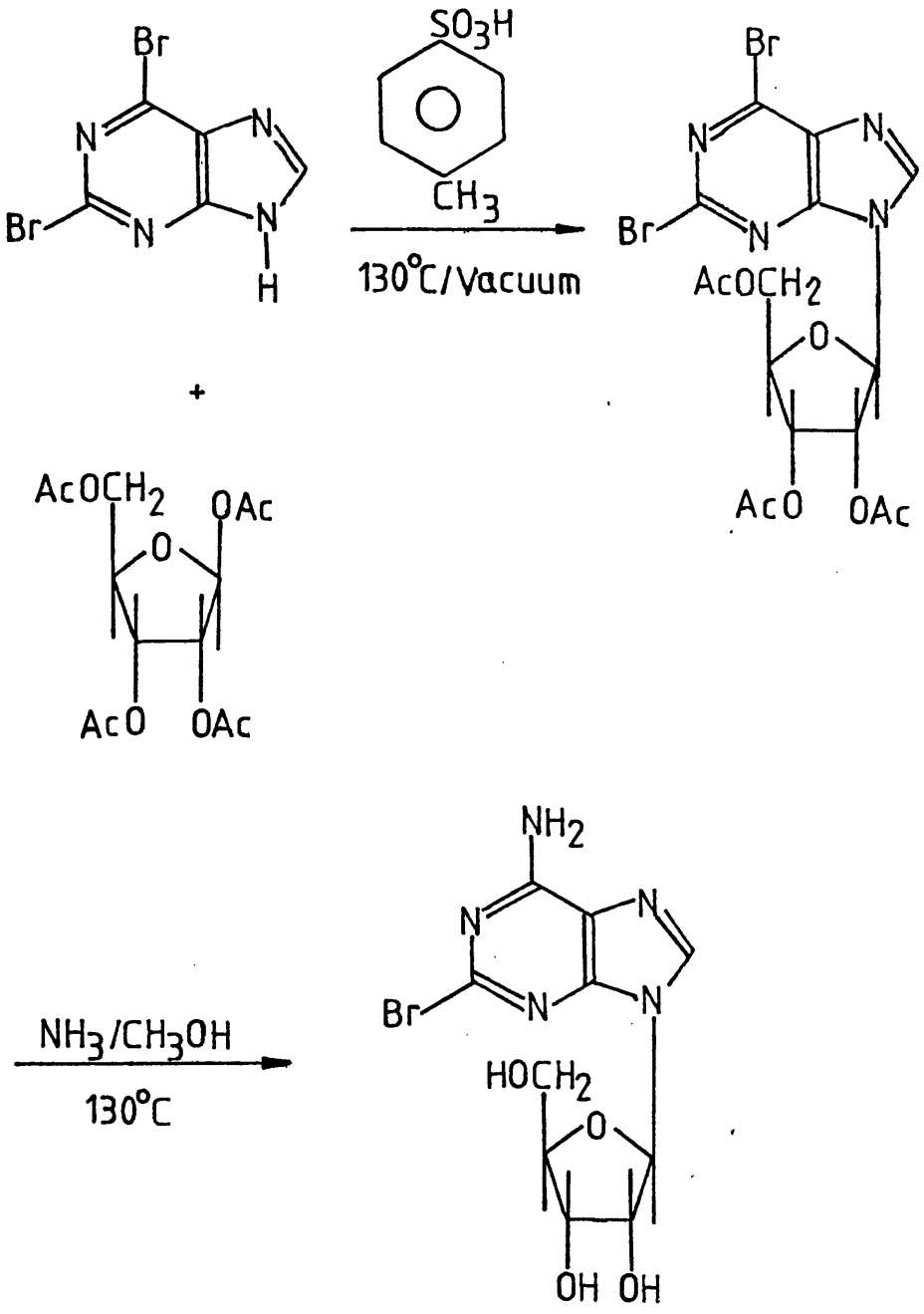
Imai and his co-workers (1966) have shown that the fusion of 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribose with various purine derivatives in the presence of iodine affords a somewhat better yield of benzoylated nucleosides than when acidic catalysts are employed. More recently, the superiority of iodine over p-toluenesulphonic acid as a catalyst is demonstrated in the preparation of 7-(3', 4', 6'-tri-O-acetyl-2'-deoxy-β-D-arabinohexosyl) theophylline, 6-chloro and 2,6,8-trichloro-9-(2',4',6'-tri-O-acetyl-3'-deoxy-β-D-xylosyl)-9H-purine and 7-(2',4',6'-tri-O-acetyl-3'-deoxy-β-D-xylosyl)-theophylline (Antonakis and Leclercq; 1968).

The Glycal-Mercuri method consists of dissolving an acylated glycal in an inert solvent such as benzene and saturating the solution with anhydrous hydrogen chloride or bromide. The resulting product, a mixture of anomeric O-acyl-2-deoxyglycosyl halides, is freed from excess hydrogen halide and, without further purification, is added in solution to a suspension of a monopyrimidinyl mercury salt dissolved in dimethylformamide. The resulting product is a mixture of acylated anomeric 2'-deoxynucleosides, which are deacylated without resolving them at this point, affording a mixture of the α- and β- anomeric forms of the nucleoside. Often the anomers are then resolved by chromatography and recrystallisation.

The conversion of thymine into the anomers of 1-(2-deoxy-D-arabino-hexopyranosyl)-thymine (Etzold and Langen; 1968) illustrates this procedure (Fig. 24).

The method is also applicable to the preparation of thymine derivatives containing 2-deoxy-D-erythro-pentapyranosyl, 2-deoxy-L-erythro-

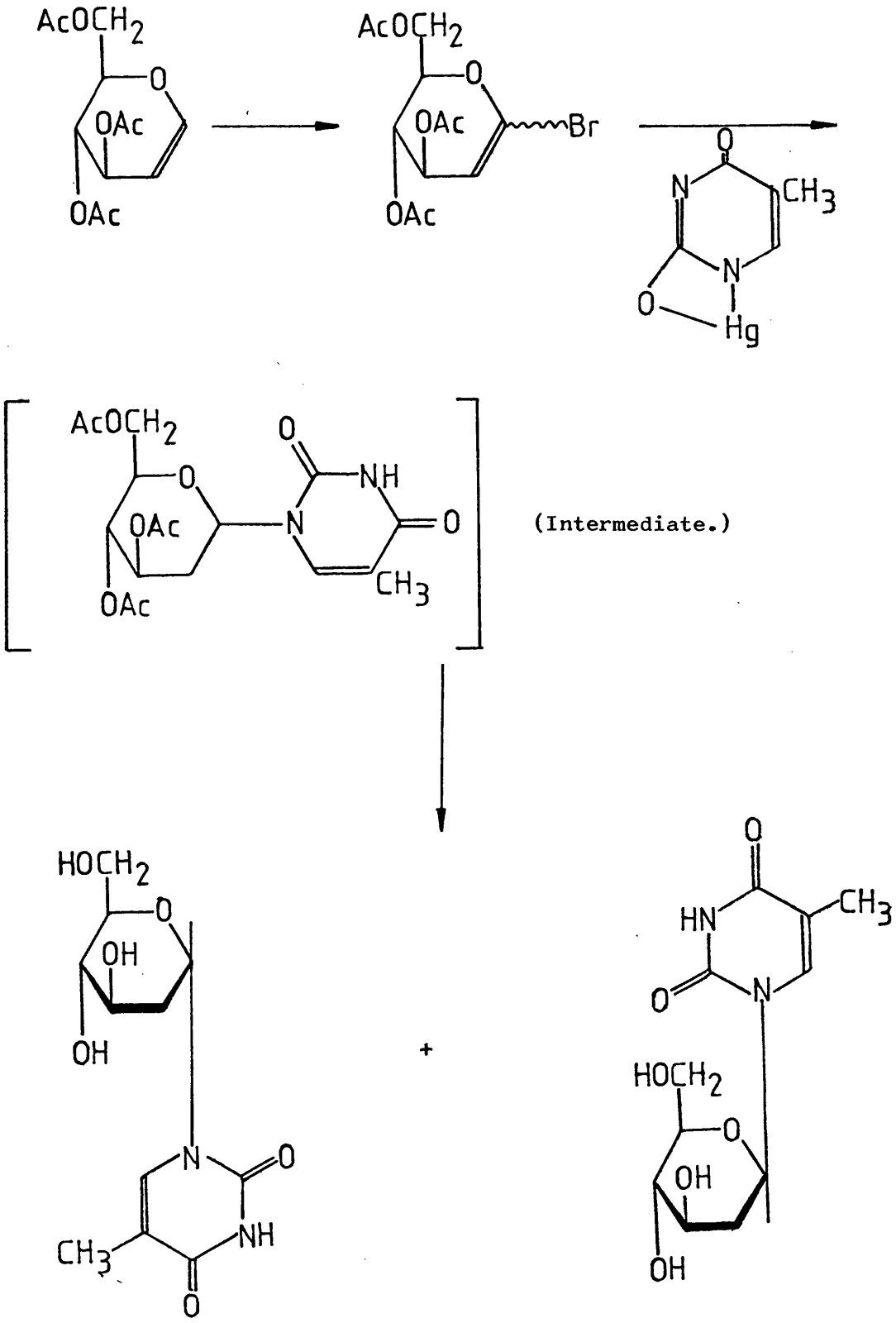
Fig. 23.



Ac = acetyl group

Synthesis of 2-bromoadenosine.

Fig. 24.



(Intermediate.)

Ac = acetyl group

Synthesis of nucleosides by the Glycal-Mercuri method.

pentapyranosyl or 2-deoxy-D-threo-pentapyranosyl residues, as well as to 2-deoxy-D-arabinohexopyranosyl nucleosides of uracil, 5-bromouracil, 5-fluorouracil and 5-iodouracil (Etzold et. al.; 1966: 1968: 1968a: Fox et. al.; 1963: Zorback et. al.; 1965: 1968). As a mixture of the anomeric O-acyl-2-deoxyglycosyl halides is employed in the condensation the formation of both the α-D- and β-D-anomers is inherant in the method.

The mercuric cyanide/nitromethane procedure introduced by Helferich and Weis (1956) is a modification of the Koenigs - Knorr synthesis of glycosides and involves the substitution of mercuric cyanide for either silver carbonate or silver oxide, both of which have been employed as routine acceptors in the synthesis of O-glycosyl derivatives (Koenigs and Knorr; 1900: 1901). By substituting nitromethane for the more usually used inert solvents the reaction may be performed in the homogeneous phase, thus leading to improved yields of acylated glycosides.

In the initial application of the mercuric cyanide/nitromethane procedure to the synthesis of nucleosides (Yamoaka et. al.; 1965) outstanding yields of per-O-acylated nucleosides derived from N-benzoyladenosine, N-benzoylcytosine, 6-chloropurine, 2,6-dichloropurine, theophylline and 2,4,8-trichloropurine were obtained using 2,3,4,6-tetra-O-acetyl-α-D-glucosyl bromide or chloride.

With the exception of amino-substituted pyrimidines, pyrimidines such as thymine and uracil may be directly subjected to the reaction, without prior conversion to the mercury derivative. In the case of bases such as adenine and cytosine it is advantageous to protect the amino group by acylation prior to coupling. However, it has been shown that 2-thiocytosine may be used directly in the reaction. Coupling of this compound with 2,3,5-tri-O-acetyl-D-ribosyl bromide affords the acetylated nucleoside in 29% yield (Rogers and Ulbricht; 1969).

Many purines and pyrimidines are not very soluble in nitromethane and so these compounds are suspended in a solution of glycosyl halide in nitromethane in order to facilitate the reaction, which, as it proceeds, becomes homogenous. This procedure is applicable to the preparation of per-O-acylated-1-α-L-arabinofuranosyl -N-benzocytosine(Yamaoka et. al.; 1968) and N-acetyl-1-(2,3,5-tri-O-benzoyl-4-O-methanesulphonyl-β-D-galactosyl)-cytosine (Watanabe and Kotick; 1969); the latter in 100% yield. The ready preparation of a number of pyrimidine nucleosides employing this method has been reported (Fox and Watanabe; 1969) and it was observed that exceptionally high yields of acylated products were obtained from pyrimidines containing highly electronegative groups at C-5. By this method the following nucleosides were prepared:-
acylated β-D-glucopyranosyl (di or tetra-hydro) pyrimidine nucleosides derived from 6-oxo-1,6- dihydro-pyrimidine, thymine, 5-ethoxycarbonyl- and 5-fluorouracil; benzoylated-β-D-ribofuranosyl (di-and tetra-hydro) pyrimidine nucleosides derived from N-benzoylcytosine, thymine, uracil or derivatives of uracils containing ethoxycarbonyl, cyano, fluoro or nitro substituents at C-5. The formation of 2',3',5'-tri-O-benzoyluridine (77% yield) is particularly noteworthy because in the standard "mercuri" synthesis uracil fails to give nucleoside products.

By far the simplest method for the synthesis of nucleosides in which a glycosyl-nitrogen bond is formed was developed by Schramm and his co-workers (1962) and takes the form of the condensation of a sugar with a base in the presence of phenyl polyphosphate. In this procedure unprotected sugars and unsubstituted purines can be used directly, thus eliminating the necessity to prepare intermediates. Because of its simplicity this method is potentially the most

powerful synthesis of nucleosides, being less time-consuming and laborious than most other methods. A further advantage is that the stereochemical restrictions of acylated sugars or acylated glycosyl halides are avoided, and that anomers, not readily accessible by other methods, can be prepared in satisfactory yield.

The method consists of heating an acidified solution of a purine, phenylpolyphosphate and a pentose in dimethylformamide at 50°C for 5 minutes to give a mixture of anomeric nucleosides.

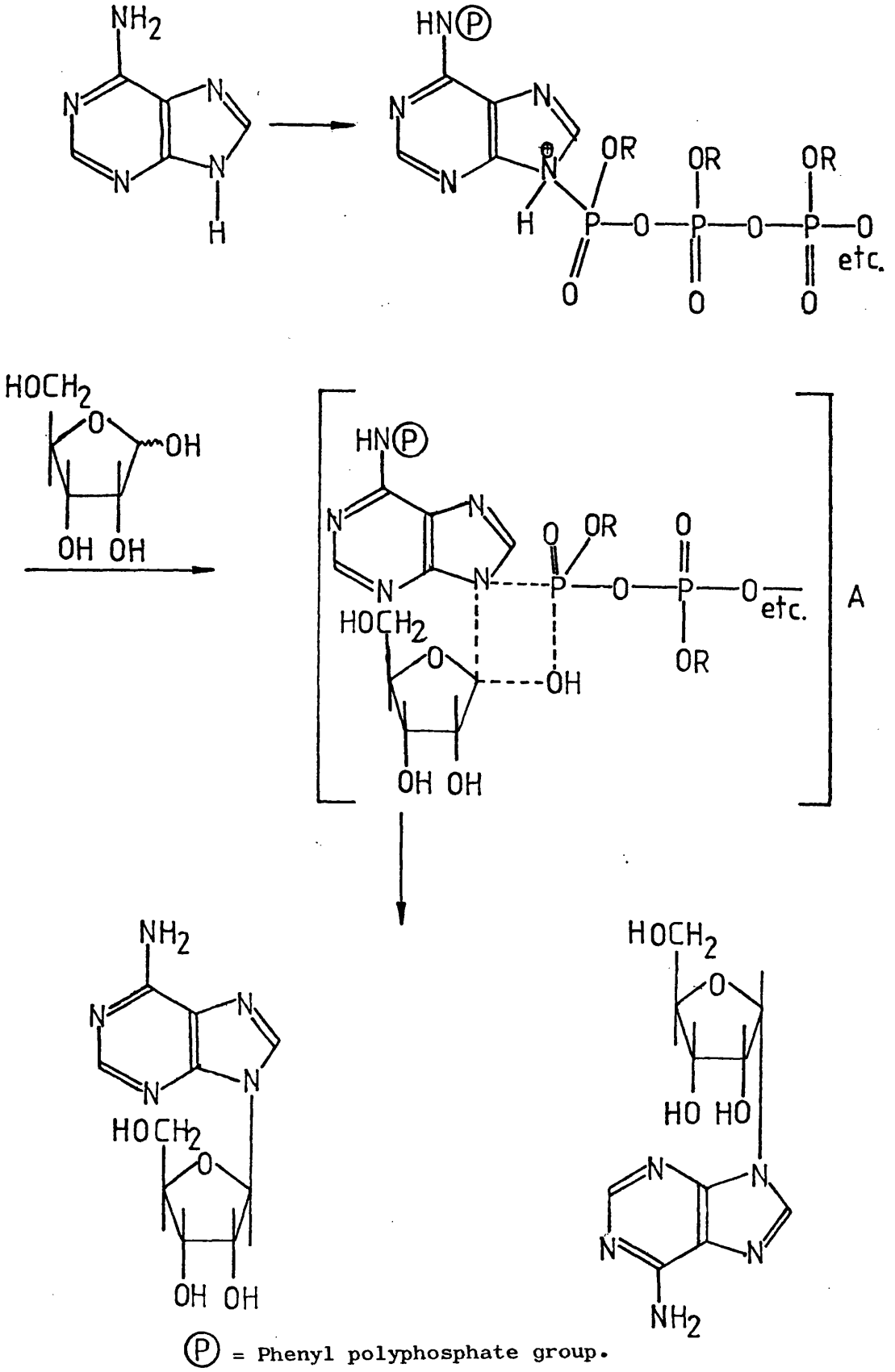
The procedure has been employed in the preparation of 2'-deoxyadenosine and 9(β-D-arabino-furanosyl)adenine (Carbon; 1963: Cohen; 1966). However, the results are difficult to reproduce.

In order to develop the procedure into a useful preparative method extensive studies have been carried out with adenine and D-ribose or 2-deoxy-D-erythro-pentose (2-deoxy-D-ribose). Thus, optimal reaction conditions were worked out and a structure for the intermediate polyphosphate ester suggested.

The synthesis of adenine and its anomer (Fig. 25) illustrates the reaction sequence. Adenine reacts with phenyl polyphosphate to afford a phosphorylated intermediate which then couples with D-ribofuranose to give the intermediate A. In acidic solution the phosphate ester residue is transferred from the 9-position of the purine to the hydroxyl group at C-1 of the pentose, thus enabling nucleophilic attack of N-9 on C-1' probably by means of a concerted reaction. The phosphoramide bond is subsequently cleaved in aqueous solution to yield equal amounts of adenosine and its anomer with a total yield of approximately 20%.

The method is applicable to the condensation of adenine with 2-deoxy-D-erythro-pentose, affording a mixture of 2'-deoxyadenosine and its

Fig. 25



(P) = Phenyl polyphosphate group.

Nucleoside synthesis using phenyl polyphosphate.

anomer with a yield of 40% (Schramm et. al.; 1967) in which the α-form predominates slightly. Similarly heating adenine and D-arabinose for 5 minutes at 50°C in the presence of phenyl polyphosphate gives a 25% yield of 9(D-arabinofuranosyl)adenine, with the α-and β-anomers in a ratio of 4:3. This method represents a simple method for the synthesis of biologically active 9(β-D-arabinofuranosyl)adenine.

Todd and his co-workers (1947b: 1950: 1957) prepared NMN with a view to the total synthesis of NAD^+ in order to verify the structure of various nicotinamide based co-enzymes. Adenosine-5'-phosphate had already been prepared (Baddiley and Todd; 1947) and so their first step was the synthesis of dihydro-nicotinamide riboside. The first synthesis of a glycosyl-pyridinium salt was that of N-(2',3',4',6'-tetra-acetyl-D-glycosyl)pyridinium bromide (Fischer and Raske; 1910) which was prepared by the reaction of α-acetobromo-glucose and pyridine in the presence of phenol.

Karrer and his co-workers (1937) investigated the structure of NAD^+ and designated the nicotinamide ring as the moiety reduced during its coenzymic action. They extended the work of Fischer and Raske to the preparation of several related compounds and acetobromoglucose, acetobromoarabinose and acetobromoxylose were all condensed with nicotinamide in dioxan solution. These quarternary ammonium salts showed oxidation-reduction properties similar to those exibited by NAD^+. It was later shown that the nicotinamide-ribose linkage in NAD^+ was similar to that present in the synthetic materials. This was done by the enzymic hydrolysis of NAD^+ using sweet almond press cake, which yielded adenosine and nicotinamide nucleoside which, although devoid of co-enzymic activity exhibited properties similar to those shown by Karrer's model nucleosides (Schlenk; 1940: 1941: 1942: 1943: Schlenk and Gingrich; 1942: 1944).

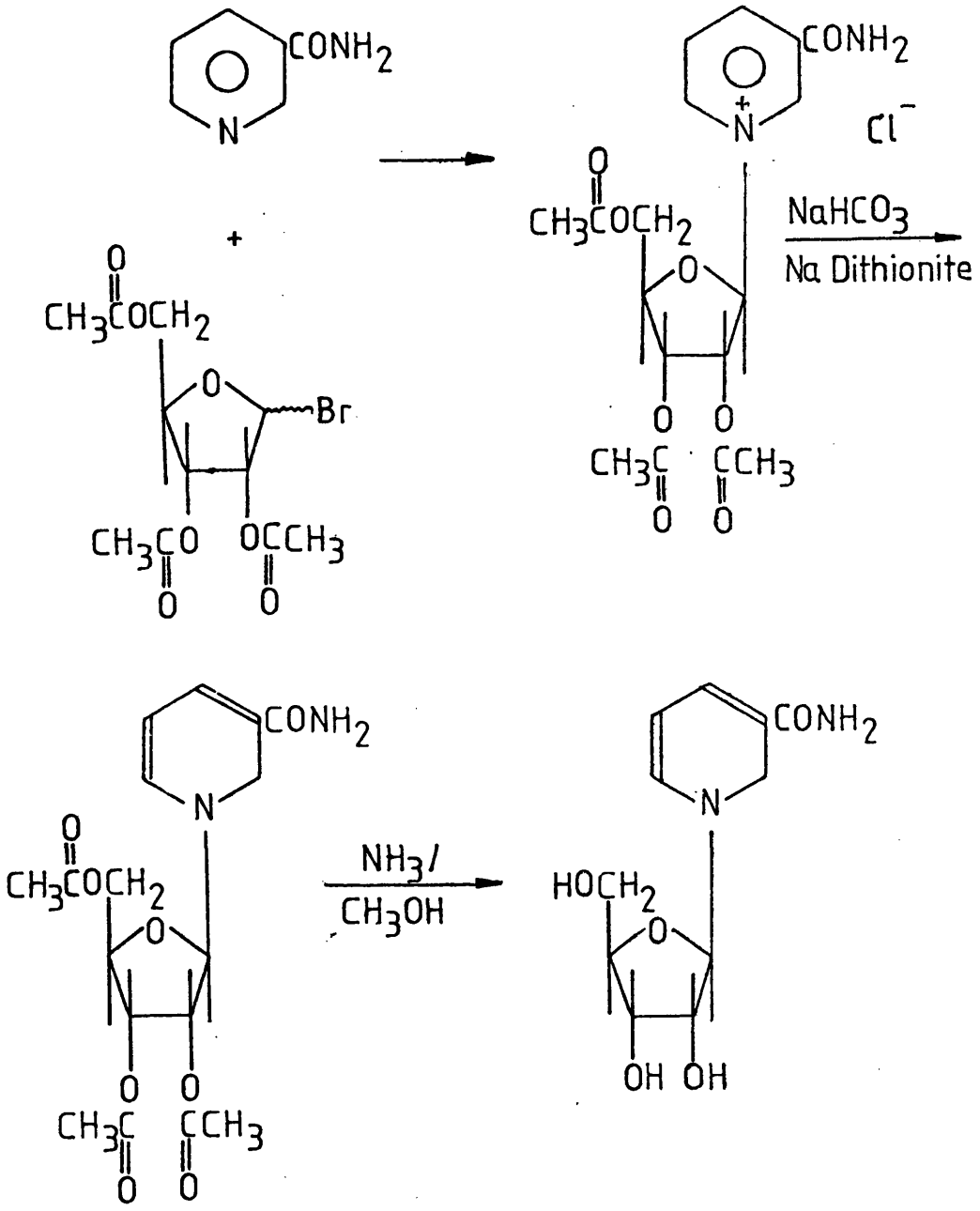
Todd further extended Fischer's work to include the preparation of quarternary ammonium salts from nicotinamide and acetohalogeno-derivatives of D-mannopyranose, D-galactopyranose and D-ribofuranose, as well as repeating the work of Karrer.

The general method used by Todd was to condense the acetylated halogenosugar with nicotinamide in dry acetonitrile at $0^{o}C$. The crude product was purified by dissolving in chloroform and reprecipitation by the addition of diethyl ether. The quarternary ammonium salt was dissolved in saturated aqueous sodium hydrogen carbonate and the solution was saturated with carbon dioxide and sodium dithionite added. The product was extracted with chloroform and isolated by the addition of petroleum spirit, finally being deacetylated by dissolution in dry ammonia-saturated methanol. (Fig.26).

Because of the great instability of N-(D-ribofuranosyl)-1,2-dihydronicotinamide obtained from the reaction of nicotinamide and 1-bromo-2,3,5-tri-O-acetylribofuranose this glycoside was unsuitable as the starting point of the synthesis of NAD^{+}. Hitherto ammoniacal deacylation of the quarternary glycosides had not been regarded as a satisfactory step in nucleoside synthesis because of the instability of the nucleoside under alkaline conditions.

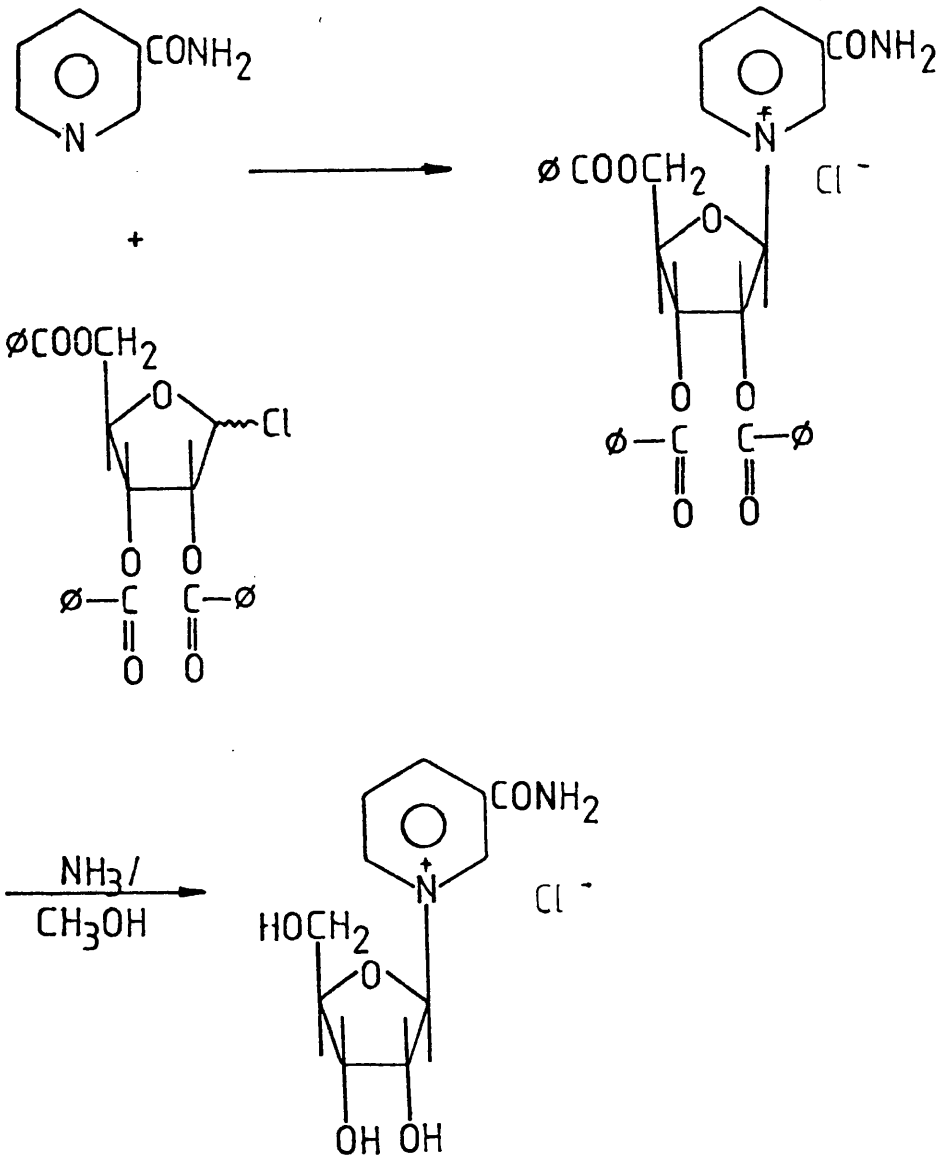
Consequently acidic hydrolysis was widely used until Todd discovered that ammoniacal hydrolysis of quarternary ammonium salts could be performed with care. Todd improved the preparation by using 1-chloro-2,3,5-tri-O-benzoylribofuranose as the glycoside, condensing this with nicotinamide to form N-(2',3',5'-tri-O-benzoyl-D-ribofuranosyl)-3-carbamoylpyridinium chloride which was debenzoylated by dissolution in dry ammoniacal methanol to give N-(D-ribofuranosyl)-3-carbamoyl-pyridinium chloride, the intermediate compound in the synthesis of NMN (Fig. 27). It would seem that this method could be adapted to the synthesis of 3-trifluoroacetylpyridine nucleoside.

Fig. 26.



Synthesis of N-(D-ribofuranosyl)-1,2-dihydronicotinamide.

Fig. 27.



Ø = phenyl

Synthesis of nicotinamide nucleoside

The effect of acylated α-bromosugars giving exclusively glycoside with β-configuration is readily explicable on the assumption that the reaction proceeds with a single, normal inversion at C-1 of the sugar. However, the synthesis of the natural β-nucleosides described all used syrupy ribofuranosyl halides which are almost certainly a mixture of the α- and β- forms. However, in the synthesis of puromycin (Baker et. al.; 1955) a crystalline halogenosugar known to have the β-configuration also gave only the β-nucleoside. Baker (1957) suggested that the condensation of a heavy metal salt of a purine with an acylated glycosyl halide will form a nucleoside with a 1,2-trans configuration in the sugar(i.e. usually a β-configuration) regardless of the original configuration at C1-C2. If the halogenosugar has the 1,2-cis-configuration, the purine will enter a single SN2 reaction with inversion at C-1, giving a nucleoside with the 1,2-trans configuration, which may be α- or β- depending on the sugar (Fig. 28a).

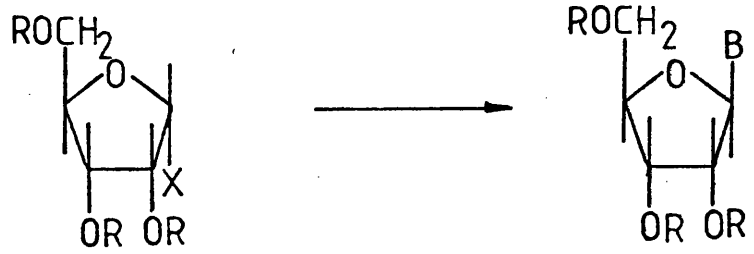
Halogenosugars with a 1,2-trans configuration undergo two SN2 reactions, the first of which is intramolecular with the participation of the 2-acyloxy group (Fig. 28b).

Thus the formation of β-ribosides from β-ribose is seen to depend on the neighbouring group participation of the 2-acyloxy group. If such a group is missing or sterically hindered a mixture of products is to be expected. This was found, for example, in the adenosine synthesis using 5-O-benzoyl-D-ribofuranosyl bromide-2,3-cylic carbonate, which gave the α- and β- anomers (Wright et. al.; 1958) and in the synthesis of 2'-deoxyribosides from 2-deoxyribose.

The synthetic NMN prepared by Todd and his co-workers had an optical rotation ($[\alpha]_D$) of-38° which was compatible with the β-anomer, whilst the optical rotation of the α-anomer, obtained from an

Fig. 28.

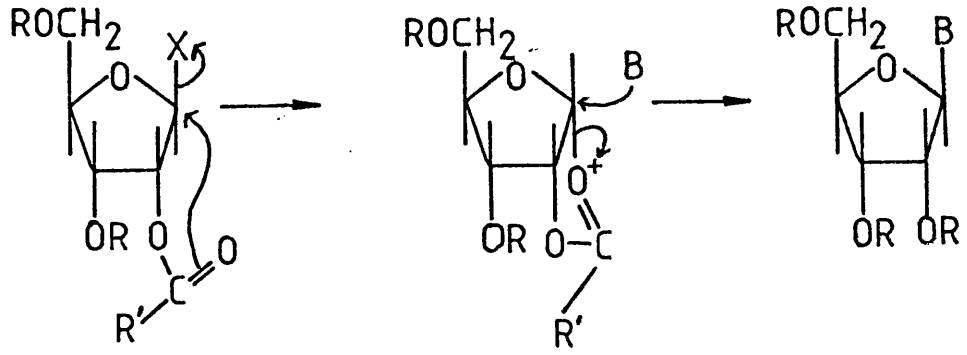
(a) Glycoside formation when the halosugar has a C1-C2 cis configuration.



β-D-riboside

α-D-arabinoside

(b) Glycoside formation when the halosugar has a C1-C2 trans configuration.



R = benzoyl; R' = phenyl; X = halide; B = base.

impurity in NAD^+, has an optical rotation of $+58^o$.

Nucleotide synthesis

The last stage in the preparation of a nucleotide is the phosphorylation of the nucleoside. A great range of reagents have been used with varying degrees of success. In their phosphorylation Todd and his co-workers encountered several difficulties, the first being that nicotinamide riboside is insoluble in most organic solvents and sufficiently unstable to render impractical any protection for the 2' and 3' hydroxyls with, for example, an isopropylidene residue. A solution would be to prepare the nucleoside from a ribofuranose containing a reactive group, such as iodo, or a protected phosphoryl residue in the 5'- position. However, Todd (working in collaboration with R L Hinman) met with little or no success, and so this approach was abandoned.

Todd then turned to direct phosphorylation of the nucleoside using, firstly, phosphoryl chloride in moist pyridine, a reagent that had been used successfully in the field of riboflavin chemistry (Forest and Todd; 1950). However, despite initial success it was found that nicotinamide nucleoside had a half-life of only eighteen hours in moist pyridine, being converted into another nucleoside later identified (by paper chromatography) to be quarternary pyridine ribofuranoside (indicating a transglycosylation between pyridine and nicotinamide). The nicotinamide nucleoside was, however, stable when dissolved in anhydrous m-cresol or dimethyl formamide, or when suspended in acetonitrile or nitromethane. Nitromethane was found to be the best medium for phosphorylation, although acetonitrile was almost its equal. The nucleotide was isolated by passage down anion and cation exchange resins. It was found that the nucleoside had been phosphorylated to an extent of 20%, and was exclusively the 5'-phosphate. This was proven by hydrolysis with snake vemon 5'-nucleotidase (which caused the complete

breakdown of the nucleotide), periodate titration and both chromatography and electrophoresis on paper in the presence of borate ions. Moreover, the paper chromatographic behaviour was identical to that of an authentic sample of NMN obtained by enzymic degradation of NAD^+. The optical rotation ($[\alpha]_D = -24^o$) showed that the anomers were present in the ratio of α: β:: 4:1, and this was later confirmed by further synthetic work.

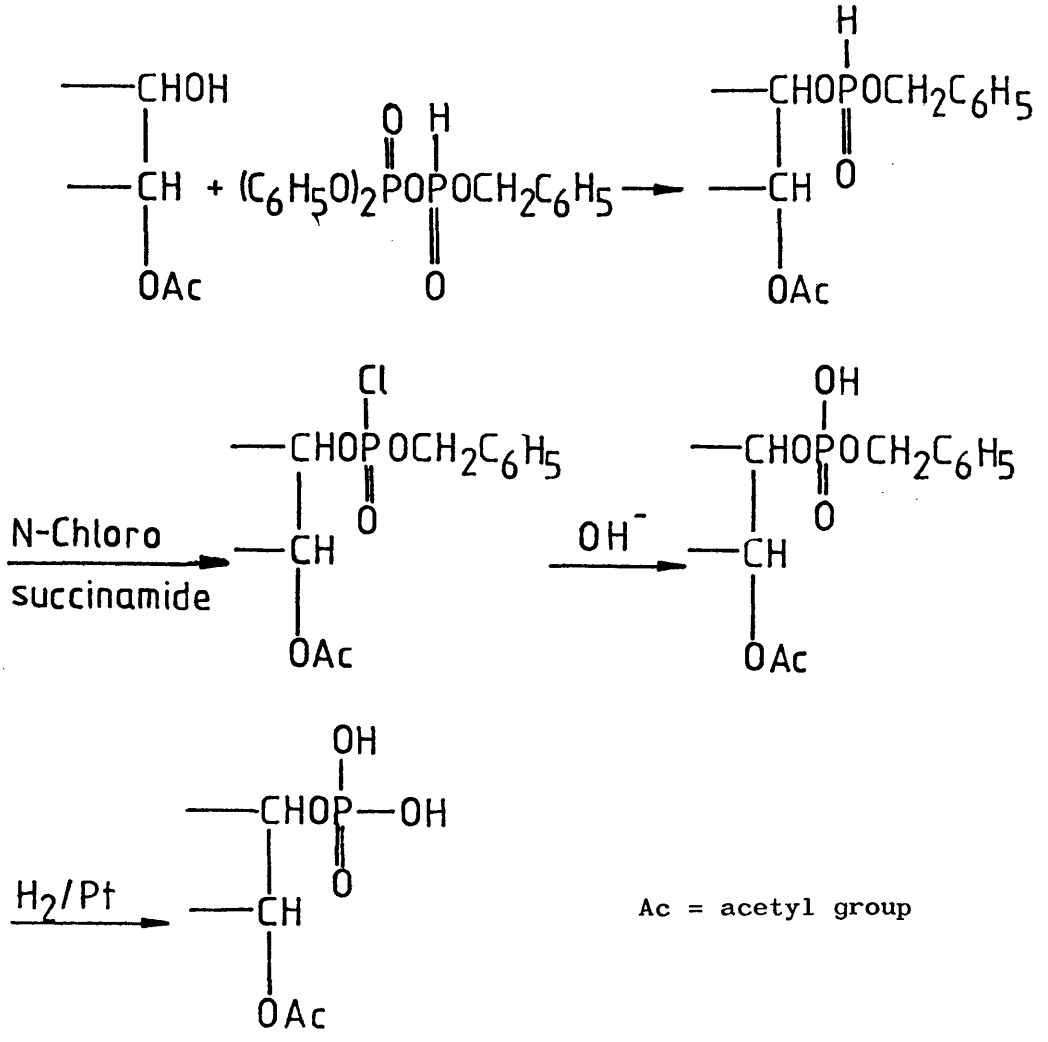
A phosphorylating agent, to be of general use must be available on a large scale, stable for long periods, very reactive and such that the unprotected intermediates can be converted into the desired products under mild conditions. Many reagents have been suggested as useful phosphorylating agents, but few fulfill all the above criteria.

O-Benzyl phosphorus-O,O-diphenyl phosphoric anhydride, in the presence of 2,6-lutidine as a catalyst, reacts with the free hydroxyl group of a sugar to form a benzyl phosphate, which can be chlorinated with N-chlorosuccinamide. Hydrolysis gives the benzyl hydrogen phosphate, and the benzyl group can then be removed by catalytic hydrogenation (Brown et. al.; 1954) (Fig. 29).

2-Cyanoethylphosphate, prepared by the reaction of phosphorus oxychloride and 1-cyano-2-hydroxyethane, has been used for the synthesis of phosphomonoesters of alcohols, particularly of nucleotides (Tener; 1961). It is coupled to the alcohol using dicyclohexylcarbodiimide (DCC) as the condensing agent and pyridine as the solvent, as exemplified by the preparation of 5'-O-tritylthymidine (Fig. 30). The protective trityl group may be removed by acid hydrolysis, and very mild alkaline hydrolysis will yield the desired phosphate ester. 2-Cyanoethyl phosphate also has the advantage of being stable in aqueous conditions.

Dibromomalonamide prepared by reaction of malonamide and bromine in the presence of sodium acetate (Backes et. al.; 1921), has been used

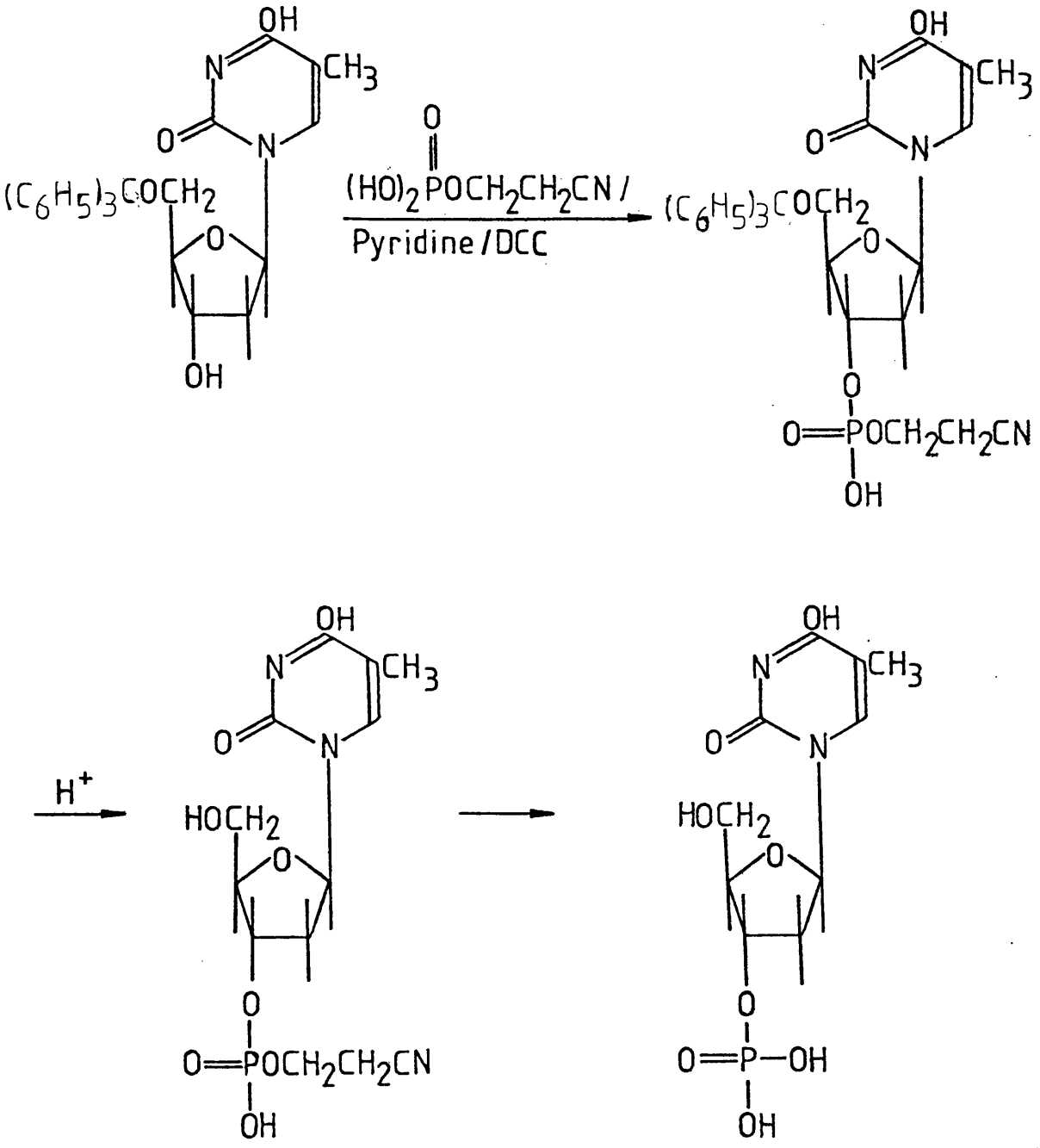
Fig. 29.



Ac = acetyl group

Phosphorylation using O-benzylphosphorus-O,O-diphenylphosphonic anhydride.

Fig. 30.



Phosphorylation using 2-cyanoethyl phosphate.

with a twofold excess of triethyl phosphite to phosphorylate hydroxy compounds (Mukaiyama et. al.; 1963).

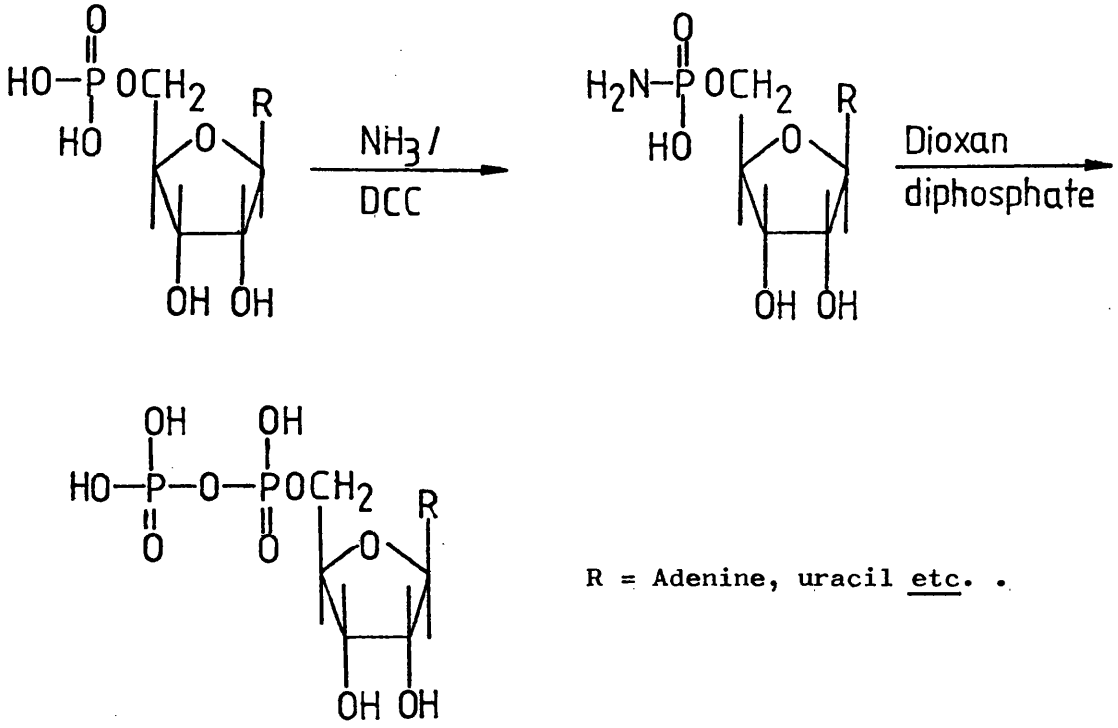
Dioxan diphosphate, prepared by the reaction of one mole of dioxan and two moles of 88% aqueous orthophosphoric acid (Baer; 1944), has been used by Chambers and his co-workers (1960) to prepare nucleoside diphosphates (Fig. 31). A nucleotide is converted into the 5'-phosphoramidate by reaction with a mixture of ammonium and DCC, and the amidate is treated with dioxan diphosphate in o-chlorophenol for three hours at 0°C. The diphosphate is precipitated by the addition of petroleum ether and purified as the ammonium salt. However, dioxan diphosphate is very hygroscopic and readily decomposed by water.

A series of diacyl phosphorochloridates (Fig. 32a) have also been used as phosphorylating agents (Brigl and Müller; 1939: Zeile and Meyer; 1938: Zervas; 1939: Atherton et. al.; 1945: Zervas and Dilaris; 1955: Witzel et. al.; 1960). These compounds may all be used to prepare phosphate ester by the same general method (Fig. 32b). Diphenylphosphorochloridate is the most widely used of these compounds and has been used in the preparation of DL-glyceraldehyde-3-phosphate (Baer; 1951). However, all the diacylphosphorochloridates have some disadvantages. They are, with the exception of the diphenyl compound, difficult to prepare on a large scale or are unstable in the presence of moisture.

Metaphosphoric acid, which is prepared by heating 85% phosphoric acid (Fig. 33a), has been used in the synthesis of pyridoxal-5'-phosphate, which was isolated as the calcium salt. Nitric acid is used in the hydrolysis step to destroy the N,N-dimethyl-glycyl hydrazine (Viscontini et. al.; 1951) (Fig. 33b).

o-Phenylene phosphorochloridate is prepared from catechol and phosphorus pentachloride, the product being reacted with acetic anhydride (Fig. 34a). The reagent reacts with a primary alcohol in the presence of base (e.g.

Fig. 31.

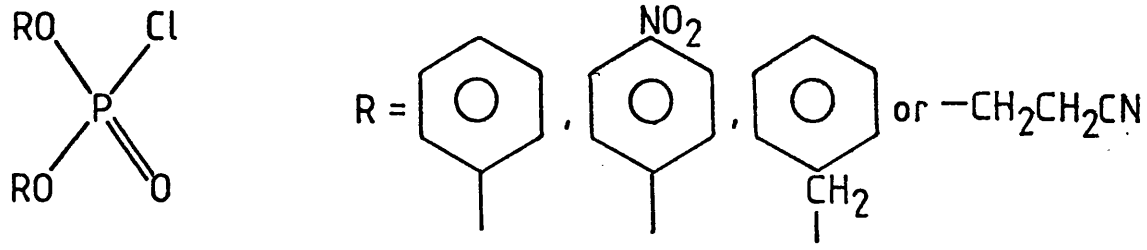


R = Adenine, uracil etc. .

Phosphorylation using dioxan diphosphate.

Fig. 32.

(a) Diącyl phosphorochloridates.



(b) Phosphorylation using the above compounds.

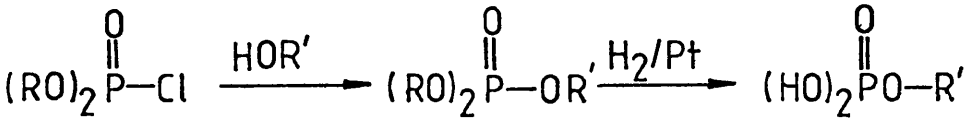
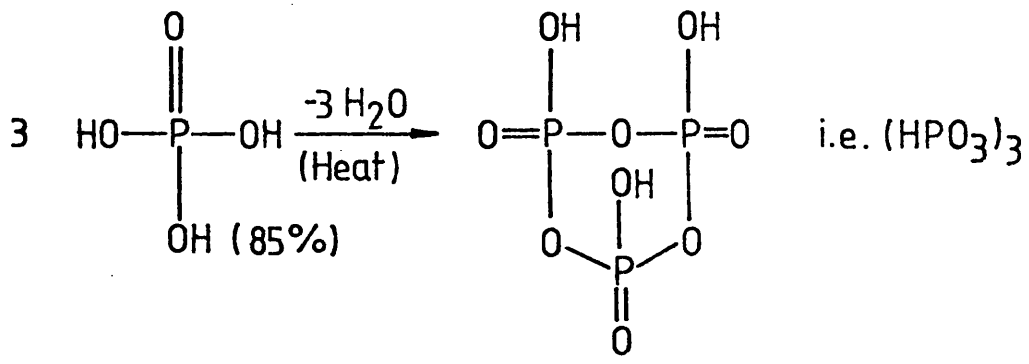
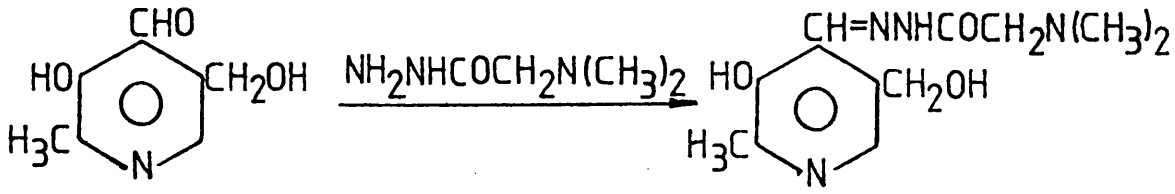


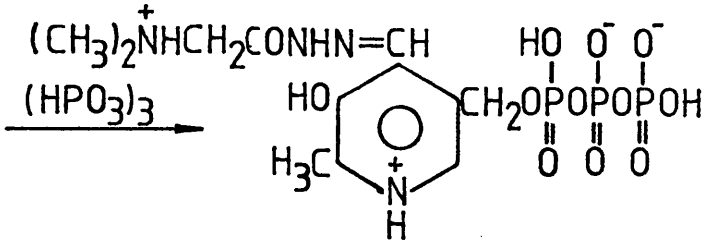
Fig. 33.

(a) Synthesis of metaphosphoric acid.



(b) Phosphorylation using metaphosphoric acid.





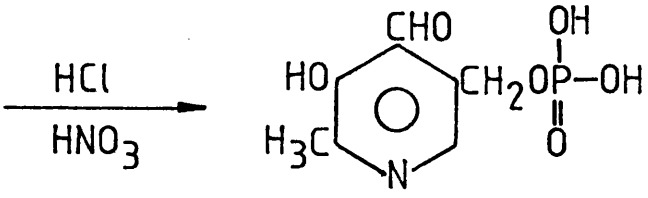
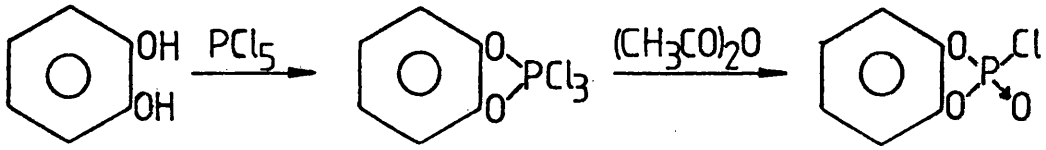
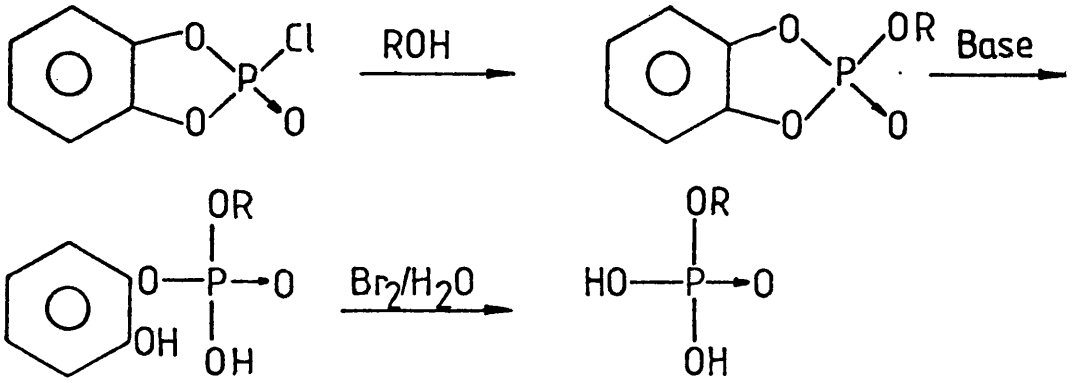


Fig. 34.

(a) Synthesis of o-phenylene phosphorochloridate.

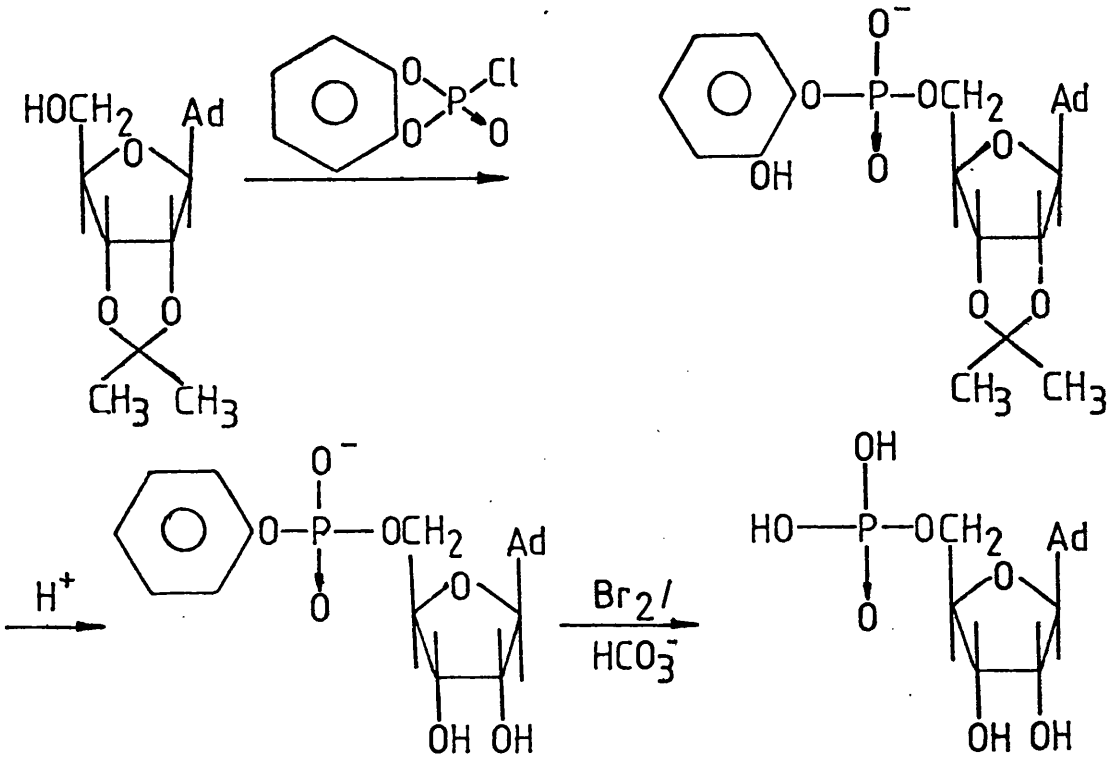


(b) Reaction of alcohols with o-phenylene phosphorochloridate.



R = acyl gpoup.

(c) Phosphorylation of adenosine with o-phenylene phosphorochloridate



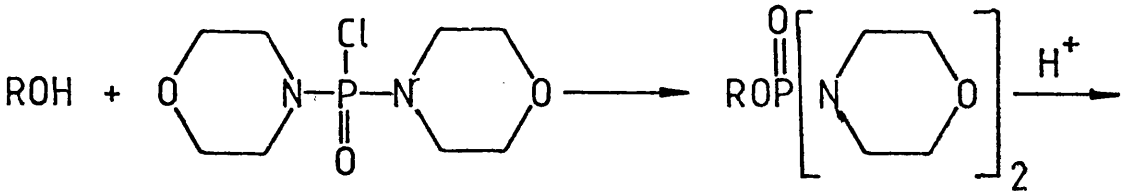
Ad = Adenine group.

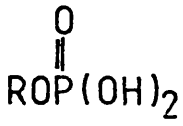
pyridine) to give a phosphotriester, which is easily hydrolysed to the o-hydroxyphenol phosphate ester which, when treated with excess bromine water, is converted into the monophosphate in good yield (Khawaja and Reese; 1966) (Fig 34b). These conditions are mild and the reactions proceed in good yield; the reagent is easily made on the molar scale, and is stable when kept in a sealed tube. Hence all criteria for a good phosphorylating agent are apparently satisfied and the reagent looks like a good one for the conversion of nucleosides into nucleotides. Khawaja and Reese (1971) have investigated the reaction between o-phenylene phosphorochloridate and the 2,3-isopropylidene derivatives of N^6, N^6-dimethyl adenosine and uridine and found the products, after isolation, to be the corresponding 5'-o-hydroxyphenol phosphates, the reaction proceeding in good yield. Acidic hydrolysis, followed by treatment with bromine in aqueous sodium carbonate oxidised the products to the corresponding 5'-nucleotide. However, complications may occur at the oxidation stage when unsaturated alcohols are phosphorylated.

Phosphorodimorpholidic chloride, prepared from morpholine and phosphoryl chloride in benzene, has been used to phosphorylate alcohols (Montgomery and Turnbull; 1958). A mixture of the alcohol, phosphorodimorpholidic chloride and 2,6-lutidine are refluxed, and the aryl diethylphosphorodimorpholidate produced is extracted with ether, and purified (Fig. 35). Hydrolysis to the dihydrogen phosphate is facilitated with a protonated ion-exchange resin. When phosphorodimorpholidic bromide, a much more reactive compound than phosphorodimorpholidic chloride, is used the reaction proceeds at room temperature.

Anhydrous phosphoric acid, prepared by dissolving phosphorus

Fig. 35.

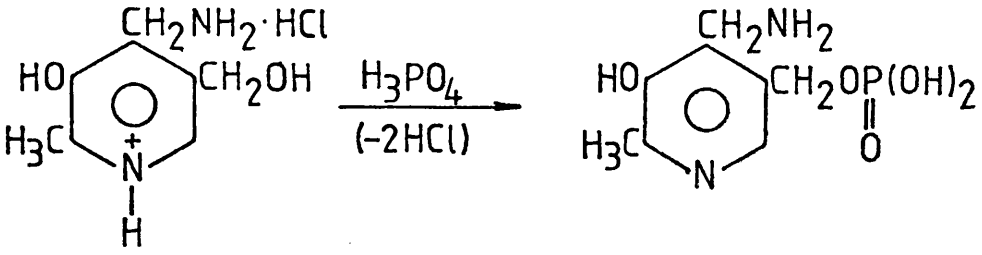


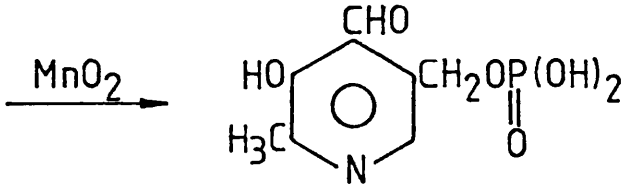


R = Acyl group.

Phosphorylation using phosphorodimorpholidic chloride.

Fig. 36.





Phosphorylation using anhydrous phosphoric acid.

pentoxide in 85% phosphoric acid, has been used as an alternative to phosphoryl chloride in the phosphorylation of pyridoxamine dihydrochloride (Wilson and Harris; 1951)(Fig. 36).

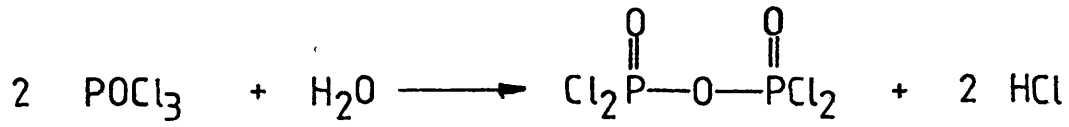
Polyphosphoric acid was used by Cherbuliez and Rubinowitz (1956; 1958) to prepare the monophosphates of various aminoalcohols and hydroxy esters, while Hall and Kharana (1955) used it for the preparation of uridine-5'-phosphate.

Pyrophosphoryl tetrachloride is prepared in 30% yield by heating phosphoryl chloride with phosphorus pentoxide in a sealed tube, and fractionating the mixture of products (Grunze; 1958). A second method involves the reaction of phosphoryl chloride and water (Besson; 1897: Becke-Goehring and Sambeth; 1957) (Fig. 37a). At low temperatures and without solvent, the reagent reacts with the primary alcohol group of 2',3'-isopropylidene nucleosides to give the 5'-dichlorophosphate which, on treatment with water gives the 5'-monophosphate (Koransky et. al.: 1962). Yields are in the order of 80% and the method avoids base catalysis and the removal of organic residues (Fig. 37b). Gruber and Lynen (1962) found that in the presence of triethylamine the reagent reacts with the free alcoholic group of 2',3'-isopropylidene adenosine to give the pyrophosphate ester. Hydrolysis and removal of the isopropylidine group with formic acid gave a material containing 22% ADP (Fig. 37c). They also showed that the reagent may be used for preparation of unsymmetrical diesters of pyrophosphoric acid and suceeded in the synthesis of coenzyme A, although the reaction only proceeded in low yield.

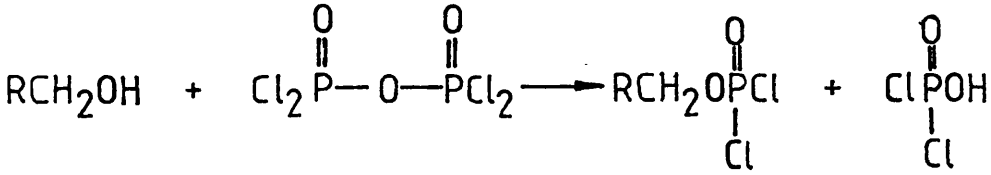
Pasternak (1950) used silver diphenylphosphate to synthesise α-D-glucose-1-phosphate (Fig.38). Tetra-O-acetyl-α-D-glucopyranosyl bromide is usually phosphorylated with inversion at C-1, but it reacts with silver diphenylphosphate with retention of configuration. The product is isolated as the crystalline potassium salt.

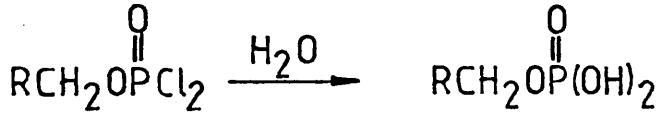
Fig. 37.

(a) Synthesis of pyrophosphoryl tetrachloride.



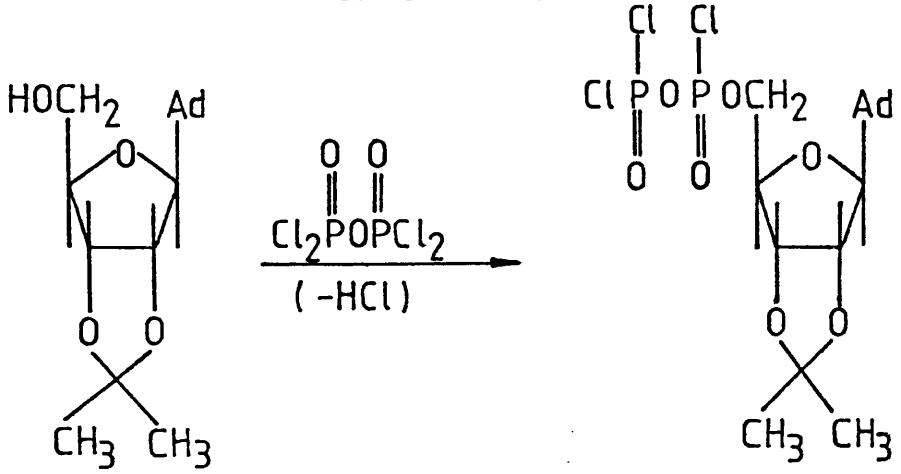
(b) Phosphorylation of alcohols by pyrophosphoryl tetrachloride.

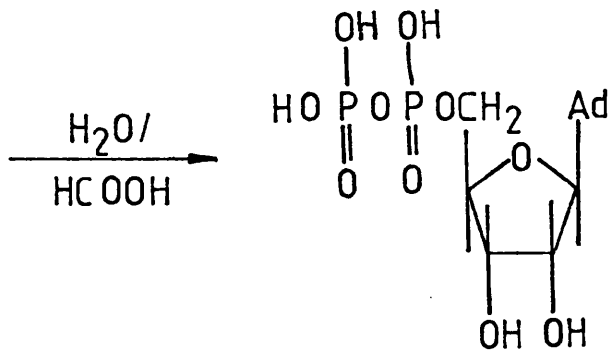




R = Acyl group.

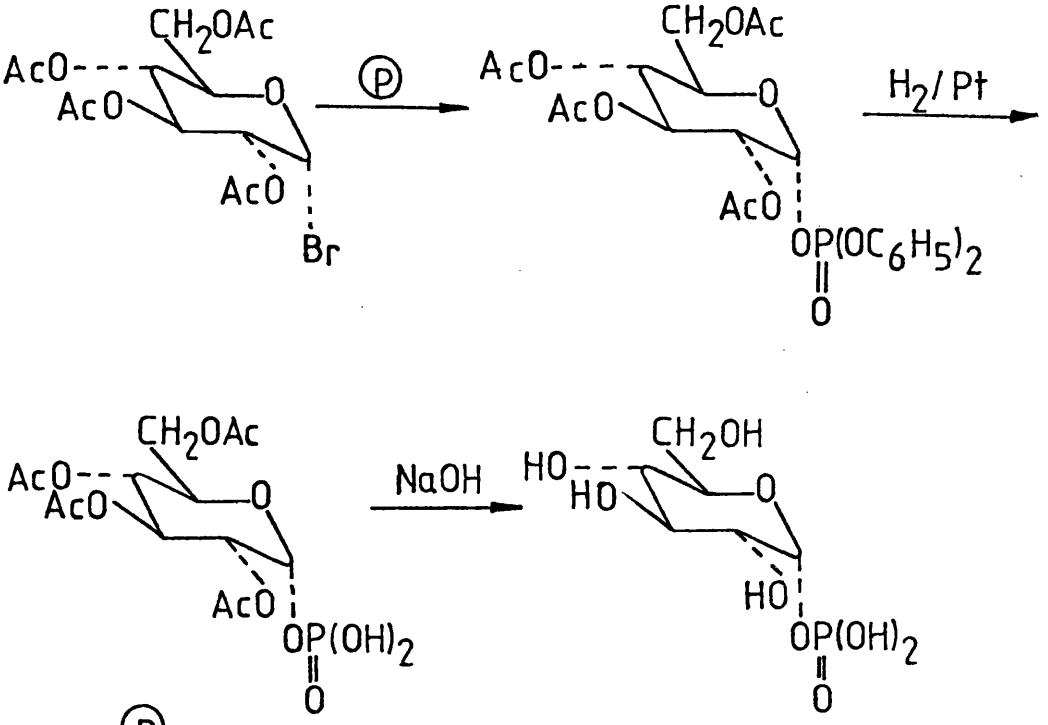
(c) Preparation of ADP using pyrophosphoryl tetrachloride.





Ad = Adenine group.

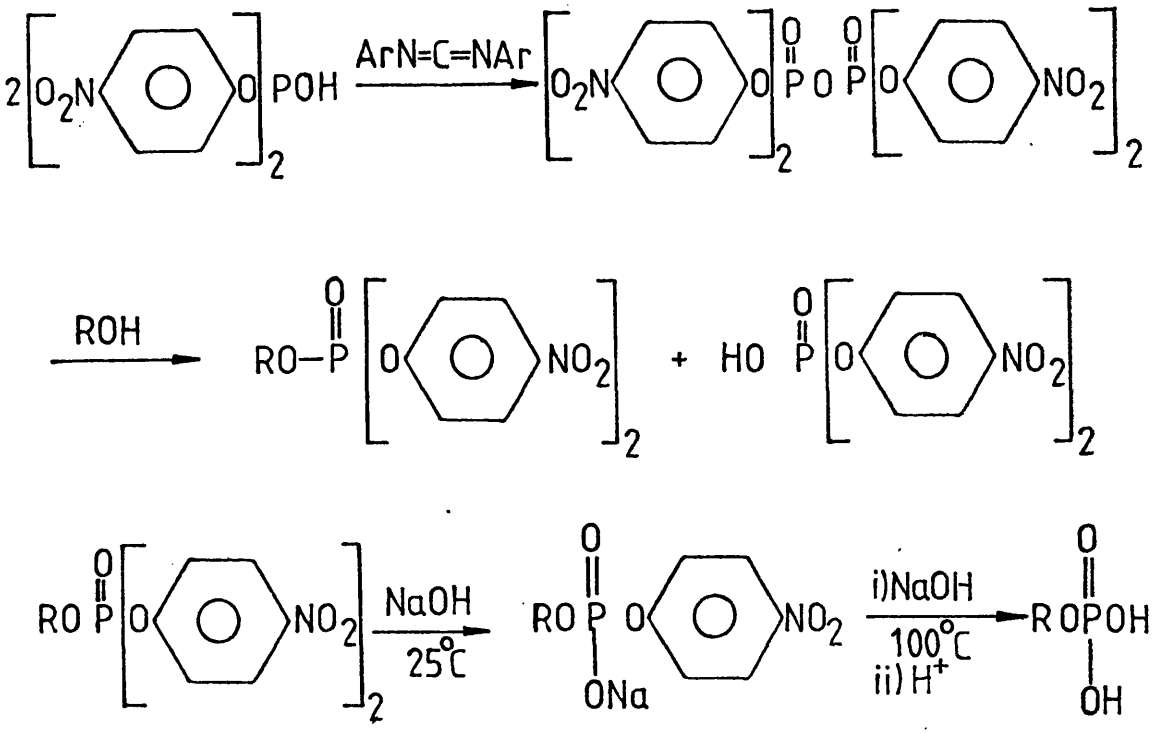
Fig. 38.



Ⓟ = Silver diphenylphosphate; Ac = acetyl group.

Phosphorylation using silver diphenylphosphate.

Fig. 39.



R = Acyl group; Ar = p-tolyl group.

Phosphorylation using tetra-p-nitrophenyl pyrophosphate.

Tetra-p-nitrophenyl pyrophosphate is prepared *in situ* by the reaction of di-p-nitrophenyl hydrogen phosphate with di-p-tolylcarbodiimide in anhydrous dioxan (Moffatt and Khorana; 1957). It reacts with an alcohol to give the neutral di-p-nitrophenyl ester without basic catalysts. One of the p-nitrophenyl groups is removed under mild alkaline conditions, the second by heating with alkali (Fig. 39). The p-nitrophenyl groups may also be removed by hydrogenolysis, the first in neutral medium, and the second in the presence of an acid catalyst. Guanosine-5'- phosphate was prepared in a yield of 70% by this method (Chambers *et. al.*; 1957).

As can be seen from the forgoing observations there would appear to be no single phosphorylating agent which fulfills all the criteria set out for the perfect phosphorylating agent and hence choice of a reactant for a particular synthesis is difficult. Hence it was decided to use o-phenylene phosphorochloridate in the synthesis of the nicotinamide riboside 5'-phosphate and if this did not succeed to follow the synthesis set out by Todd and his co-workers who, as already stated, used phosphoryl chloride at the phosphorylation step.

Chemical Methods of Pyrophosphorylation

In the original chemical synthesis of NAD^+ by Todd and his co-workers (Hughes *et. al.* 1957) the pyrophosphorylation step was facilitated using dicyclohexylcarbodiimide in aqueous pyridine, although NAD^+ had been reportedly detected enzymicly in a mixture produced by the action of trifluoroacetic anhydride on NMN and AMP. (Shuster *et. al.*; 1955). Dicyclohexylcarbodiimide had previously been used in the synthesis of nucleotide pyrophosphates in anhydrous pyridine or dimethylformamide (Christie *et. al.*; 1953), but it had also been reported (Khorana; 1954) that aqueous pyridine could be used provided that a large excess of

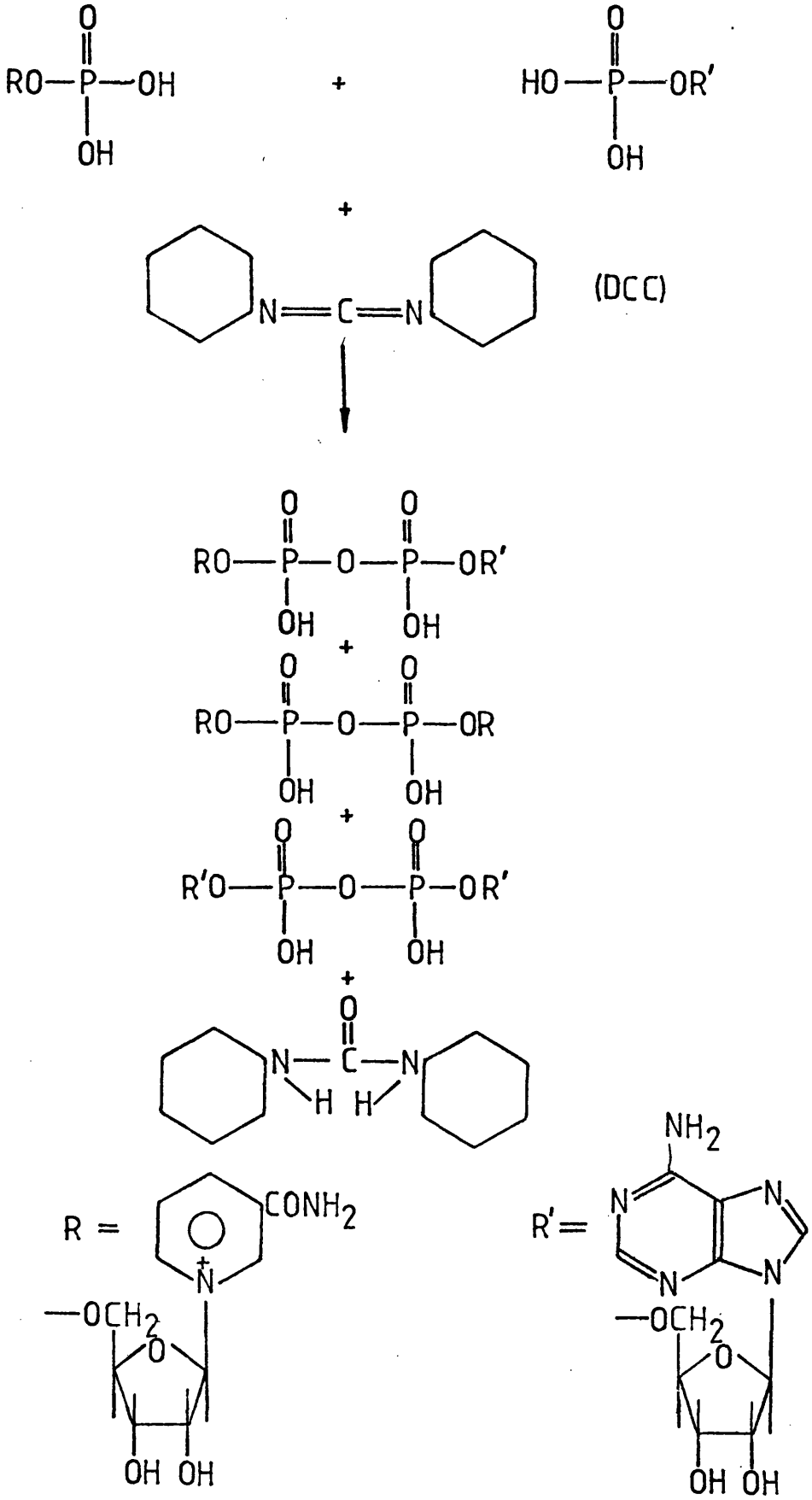
dicyclohexylcarbodiimide was used.

The expected products (Fig. 40) were obtained from the reaction, and were separated by anion exchange chromatography. The product was isolated as an amorphous powder which had the correct elemental analysis and was shown to be 70% pure β-NAD^+ (by reaction with yeast alcohol dehydrogenase). The yield of product at this stage of purification was 46%, an excellent yield for a reaction of this type. Subsequently dicyclohexylcarbodiimide, usually in aqueous pyridine, has proved to be a very useful method for the synthesis of dinucleotides, particularly in the field of NAD^+ analogues (Fig. 41). As can be seen, the reaction has not been used to prepare analogues in which the nicotinamide moiety has been replaced. This is because of the ease with which these analogues may be prepared using NAD^+ transglycosidase from pig brain. However, there is no reason why such NAD^+ analogues may not be prepared by this method.

One disadvantage with this method is that when two different nucleotides are condensed more than one product will be obtained (Fig.40). For example when the reaction to condense NMN and AMP was investigated by Hughes, as well as NAD^+, the desired product, the condensation product between two molecules of NMN, as well as the product from the condensation of two molecules of AMP were isolated. Hence, after a lengthy synthesis to prepare a dinucleotide analogue considerable product may be lost when these unwanted by-products are formed.

However, Moffatt and Khorana (1961) reported N, N'-dicyclohexyl-4-morpholinocarboxamidine, the condensation product between morpholine and dicyclohexylcarbodiimide will condense with an equimolar amount of one of the nucleotides to give the morpholidate, and then this will condense with an equimolar amount of the second nucleotide (Fig. 42). The reaction is performed in anhydrous pyridine, the product being

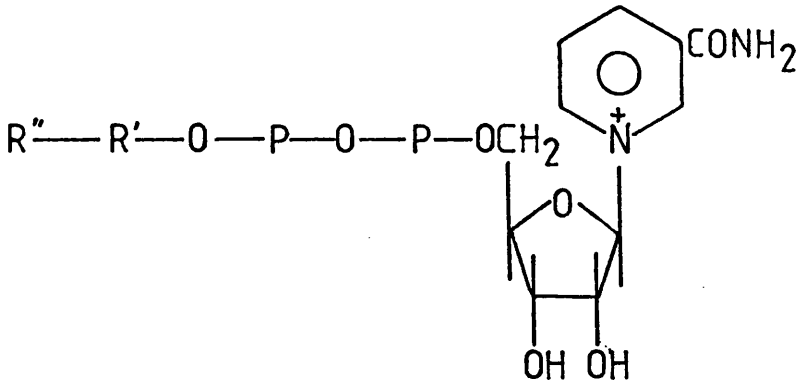
Fig. 40.



By-products obtained during the chemical pyrophosphorylation of NMN and AMP using DCC.

Fig. 41.

Some dinucleotides which have been prepared using DCC at the pyrophosphorylation stage.



R' = ribofuranose R'' = guanine

R' = ribofuranose R'' = cytosine

R' = ribofuranose R'' = uracil

R' = ribofuranose R'' = thymine

(Honjo et. al. ; 1962.)

R' = ribofuranose R'' = thiopurine

R' = ribofuranose R'' = methylthiopurine

R' = ribofuranose R'' = purine

(Pfleiderer et. al. ; 1963; 1964.)

R' = ribofuranose R'' = phenyl

(Woenckhaus and Volz ; 1966.)

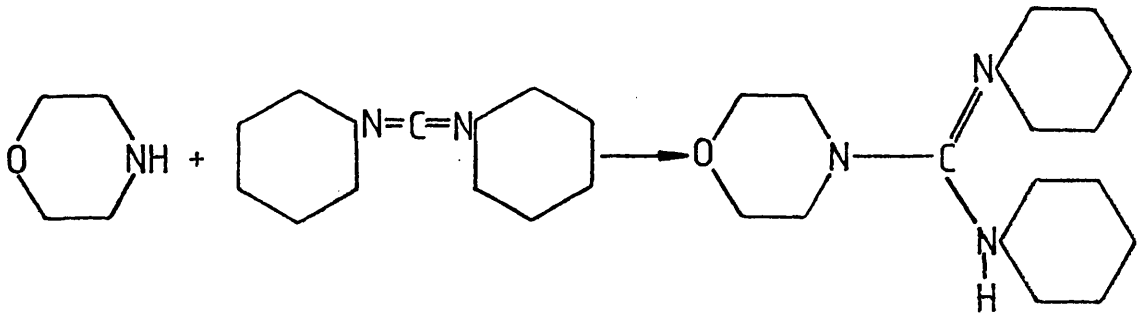
R' = ribofuranose R'' = deazapurine

(Woenckhaus and Zumpe ; 1968.)

R' = 4-thio-D-ribofuranose R'' = adenine

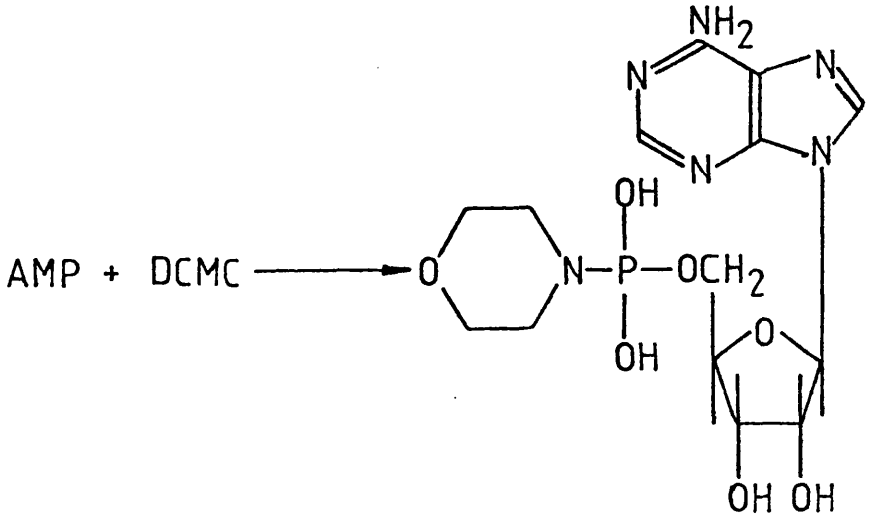
(Hoffmann et. al. ; 1970.)

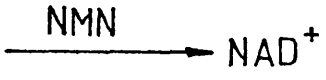
Fig. 42.



(DCMC)

Synthesis of N,N'-dicyclohexyl-4-morpholinocarboxamidine.





Pyrophosphorylation using N,N'-dicyclohexyl-4-morpholinocarboxamidine.

purified by ion-exchange chromatography. The reactions proceed in good yield, but it is possible that the 3',5'ribosyl cyclic phosphate may be prepared from the morpholidate in the presence of excess dicyclohexyl-carbodiimide, although elevated temperatures are generally required for the reactions to proceed via this pathway (Moffatt and Khorana; 1961a). Another advantage of this method is that the morpholidates are usually soluble in anhydrous pyridine, whereas the unsubstituted 5'-nucleoside phosphates are not, anhydrous pyridine being the solvent in which the reaction proceeds with the best yield (Smith et. al.; 1961).

Enzymic synthetic methods.

Dinucleotide synthesis.

Of the enzymic methods for the synthesis of analogues of NAD^+ the most attractive is that of Kaplan and his co-workers (1954) who investigated the effect of isonicotinic acid hydrazine (INH) on NAD^+ transglycosidase from beef spleen, and later isolated the INH analogue of NAD^+. The ability of NAD^+ transglycosidase from beef spleen to catalyse an exchange reaction between the nicotinamide moiety of NAD^+ and added nicotinamide was noted (Zatman et. al.; 1953) and it was proposed that this enzyme might catalyse group transfer reactions of a more general nature, that is the transfer of the ADPR group of NAD^+ to compounds structurally related to nicotinamide. Depending on the nature of the acceptor molecule the resulting analogue may, or may not, be active in oxidation-reduction reactions. Using purified NAD^+ transglycosidase INH (1×10^{-4}M) was found to cause 50% inhibition in the rate of disappearance of NAD^+, whereas with nicotinamide a ten-fold increase in concentration was required to cause a similar inhibition. This effect of INH is relatively specific, such compounds as nicotinic acid hydrazine, N'-methylisonicotinic acid hydrazine, nicotinic acid and isonicotinic acid having little effect. The only other compounds found

to inhibit the enzyme were isonicotinamide, the N-isopropyl derivative of INH, imidazolecarboxamide and thioazolecarboxamide. In further experiments NAD^+transglycosidases from other animal tissues were used under similar conditions with varying results (Fig. 43). On the basis of these results, Zatman divided NAD^+ transglycosidases into the categories of INH sensitive and INH insensitive enzymes, depending on whether the enzyme was inhibited by INH. He then looked for evidence of analogue formation in both systems, and was able to detect it in only the INH insensitive enzymes which included those from human spleen and prostrate, and horse and pig brain.

In a subsequent investigation Zatman and his co-workers (1954) reported that, using NAD^+ transglycosidase from pig brain,the INH analogue of NAD^+ was isolated in 75% yield. It was analysed chemically and the results were compatible with the analogue's having a structure similar to NAD^+, but with the nicotinamide moiety replaced with INH. Physical and enzymic properties of the analogue were reported, the analogue being found to be more than twice as potent an inhibitor as free INH of the INH-sensitive NAD^+ transglycosidases. No inhibitory action on any of the NAD^+-linked dehydrogenases was noted. In this paper it was also suggested that the mode of action is <u>via</u> an ADPR-enzyme intermediate (Fig. 44). The non-competitive nature of the inhibition of NAD^+ transglycosidase from beef spleen can readily be accounted for in this formulation by including complexes between the ADPR-enzyme complexes and the ribosyl acceptors (ADPR-enzyme---- nicotinamide and ADRP-enzyme---- INH). In the "insensitive" enzyme system the latter complex would break down to form the analogue (Fig. 44a), whereas in the case of "sensitive" enzyme systems the reaction would not proceed in either direction. Zatman believed this to account for the inhibition of NAD^+ breakdown by INH and its dependance on NAD^+ concentration; the inhibition, by INH, of the incorporation of

Fig. 43.

Effect of various inhibitors on NAD^+ transglycosidases from various sources.

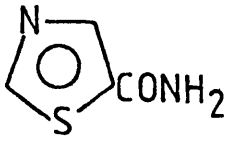
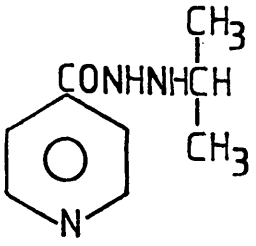
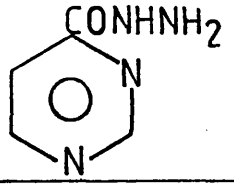
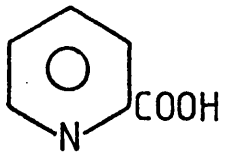
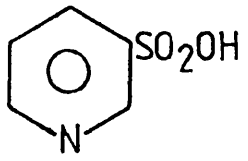
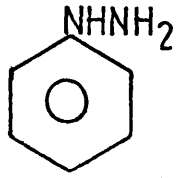
Compound	Source of enzyme	Concentration(M)	Inhibition(%)
	Beef spleen	5×10^{-3}	84
	Beef brain	1×10^{-3}	85
	"	2×10^{-4}	50
	Rabbit brain	1×10^{-3}	41
	Horse brain	1×10^{-3}	43
	Beef spleen	1×10^{-2}	76
	"	2×10^{-3}	50
	Duck brain	3×10^{-3}	78
	Human prostrate	2×10^{-2}	0
	Pig brain	2×10^{-2}	0
	Beef spleen	2×10^{-3}	0
	Pig brain	2×10^{-2}	0
	Beef brain	1×10^{-2}	0
	Pig brain	2×10^{-2}	0
	Beef brain	1×10^{-2}	0
	Pig brain	2×10^{-2}	0
	Beef spleen	2×10^{-2}	0
	Pig brain	2×10^{-2}	0

Fig. 44.

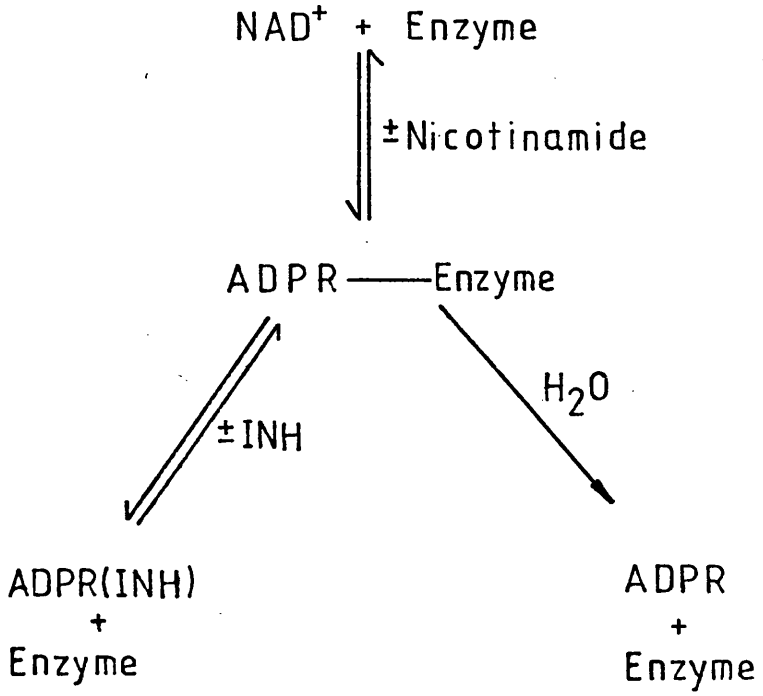


Fig. 44A.

$$\text{ADPR}\text{——}\text{Enzyme}\text{----}\text{INH} \rightleftharpoons \text{ADPR(INH)} + \text{Enzyme}$$

Formation of NAD^+ analogues using NAD^+ transglycosidase.

radioactively labelled nicotinamide into NAD^+; the failure to form detectable amounts of analogue and the failure to hydrolyse added analogue, all of which are properties of the "sensitive" enzyme system. He also reported that the INH analogue of NAD^+ is a stronger inhibitor of NAD^+transglycosidase than INH itself. This finding strengthened the view that the inhibition caused by INH is due to its conversion into the analogue, which, once formed, even in minute amounts, remains attached to the enzyme, beirg neither hydrolysed by the enzyme or released into the bulk of the medium. He also found, surprisingly, that the analogue, apart from its inhibitory action on NAD^+transglycosidases, is not a general inhibitor of NAD^+ requiring enzyme systems.

Kaplan and Ciotti (1954) reported that the NAD^+transglycosidase prepared from pig brain catalysed the formation of the 3-acetylpyridine analogue of NAD^+ from 3-acetylpyridine and NAD^+. This analogue was of particular interest, as it was the first pyridine substituted analogue of NAD^+ which was shown to be active as a coenzyme in a number of dehydrogenase reactions and 3-acetylpyridine had been shown to be an antagonist of nicotinamide in animals (Woolley; 1945: McDaniel et. al. 1955: Kaplan et. al.; 1954). In a later paper Kaplan and Ciotti (1956) compared the properties of this analogue with those of NAD^+, as well as giving the details of the synthetic method which were later shown to be general. The spectrum of the reduced form of the analogue has a λ_{max} at 365 nm with a molar extinction coefficient of 7.8, whereas NADH itself has a λ_{max} at 340 nm with a molar extinction coefficient of 6.3. The analogue is also reduced with bisulphite, and this reduced compound has an identical ultraviolet/visible spectrum to that of the enzymically reduced compound. Yarmolinsky and Colowick (1956) reported a yellow intermediate, formed during the bisulphite reduction of NAD^+. This was not thought to be the half-reduced

intermediate, but the sulphoxylate addition product, which has a λ_{max} between 355 and 360 nm. The analogue also forms a similar intermediate, which is intensely yellow, and has a distinct peak at 385 nm.

The 3-acetyl pyridine analogue of NAD^+ forms an addition product with cyanide ions, as does NAD^+ itself, the analogue, however, forming the product much easier than does NAD^+. The λ_{max} for the analogue-cyanide compound is at 340 nm whereas for NAD^+ it is 325 nm. The analogue also forms addition compounds with dihydroxyacetone and bisulphite, as does NAD^+, again the compounds adding more easily to the analogue than to NAD^+. Changes were noted in the ultra-violet/visible specta of both the analogue and NAD^+ when they were dissolved in dilute alkali. Subsequently Kaplan and his co-workers produced a series of 3-substituted pyridine analogues of NAD^+ and investigated the addition of cyanide and their reduction with yeast alcohol dehydrogenase and bisulphite. They found that, of the analogues which they investigated, only the 3-acetylpyridine and the pyridine-3-aldehyde analogues reacted with cyanide, yeast alcohol dehydrogenase and bisulphite in a manner similar to that of NAD^+. Other analogues react with none or some of these compounds. (Fig. 45)

As previously stated the method developed by Kaplan and his co-workers for the production of analogues of NAD^+ in which the nicotinamide moiety is substituted has proved to be a fairly general one. (Fig. 46)

One analogue of particular interest is the 3-chloroacetylpyridine analogue which was prepared, *via.* the 3-diazoacetylpyridine analogue, by Biellmann *et. al.* (1974). If the 3-fluoroacetylpyridine analogue could be prepared information obtained using this analogue would complement information obtained from reactions involving the 3-trifluoroacetylpyridine analogue.

The 3-chloroacetylpyridine analogue, claimed to be an alkylating agent

Fig. 45.

Comparison of the reactions of NAD^+ with some 3-substituted pyridine analogues of NAD^+.

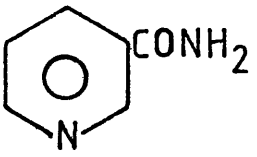
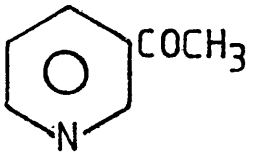
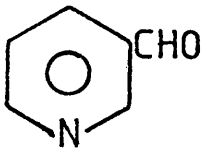
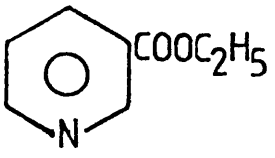
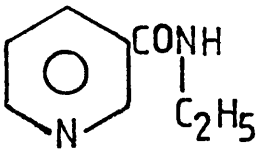
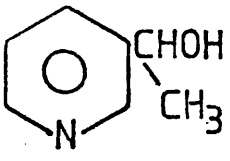
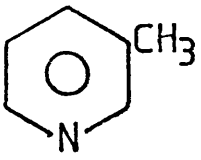
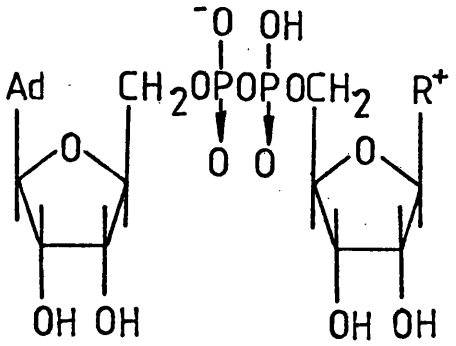
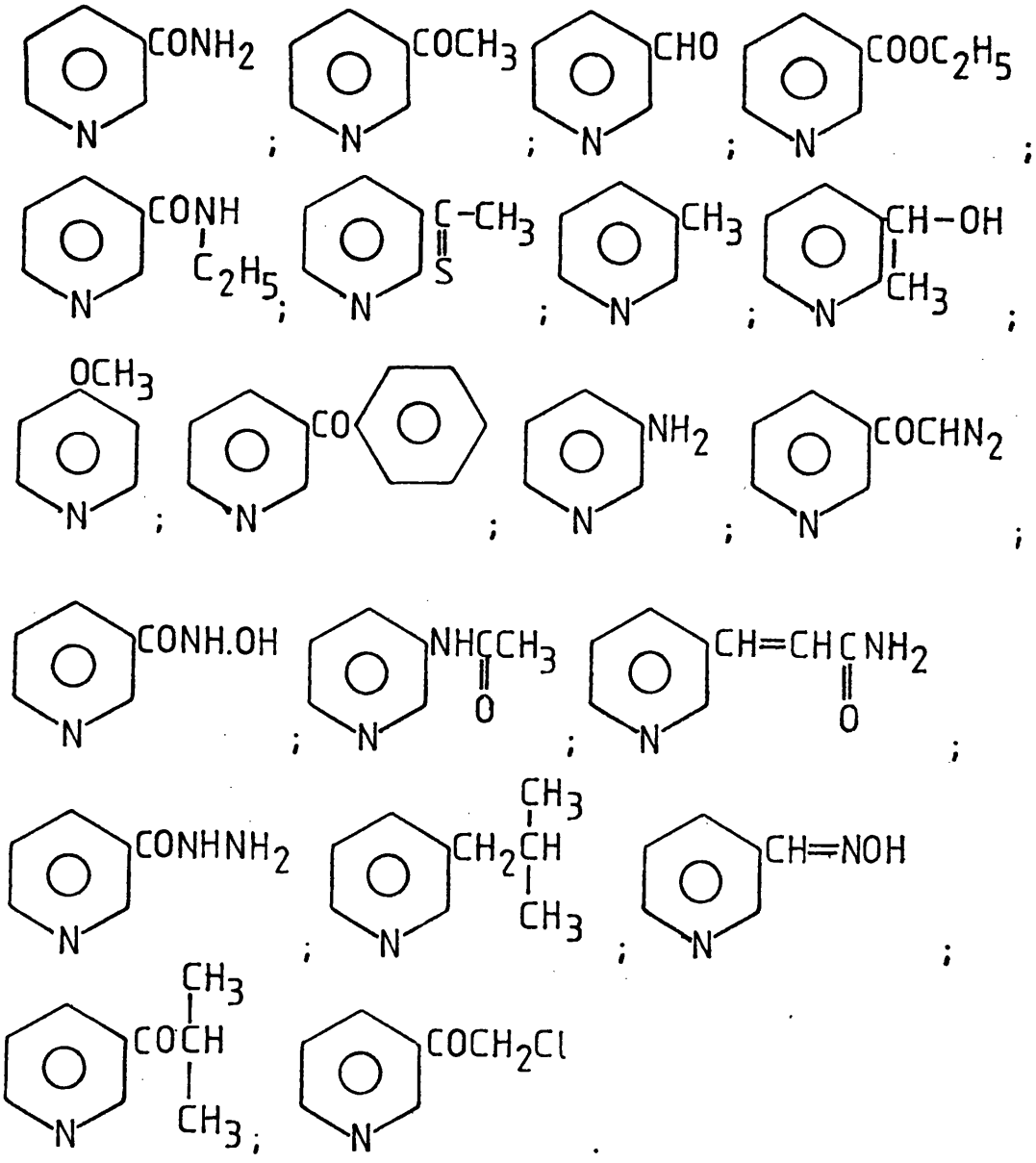
Pyridine compound	CN^- addition	Yeast ADH red^n.	Bisulphite red^n.
	+	+	+
	+	+	+
	+	+	+
	+	-	+
	+	-	+
	-	-	-
	-	-	-

Fig. 46.



Ad = Adenine; R^+ may be:-



Nicotinamide substituted NAD^+ analogues prepared using NAD^+ transglycosidase from pig brain.

can be used to detect the presence of functional amino-acid residues at the catalytic sites of dehydrogenases. As basic residues are involved in the mechanism of enzymic dehydrogenation of hydroxy compounds alkylation of these basic groups by an alkylating agent such as this analogue should yield useful information on dehydrogenation.

NAD^+ transglycosidases from animal tissue other than pig brain have been used to prepare analogues of NAD^+. For instance the NAD^+ transglycosidase from beef spleen has been used by Nishizuka et. al. (1963) and Hanjo et. al. (1964) to prepare nicotinic acid adenine dinucleotide. The transglycosidation proceeds at pH 7.5 in tris-chloride buffer and is monitored by determining the total dinucleotides by forming the cyanide adduct and then determining the NAD^+ which remains unconverted using alcohol dehydrogenase from yeast, the analogue not being reduced under these conditions.

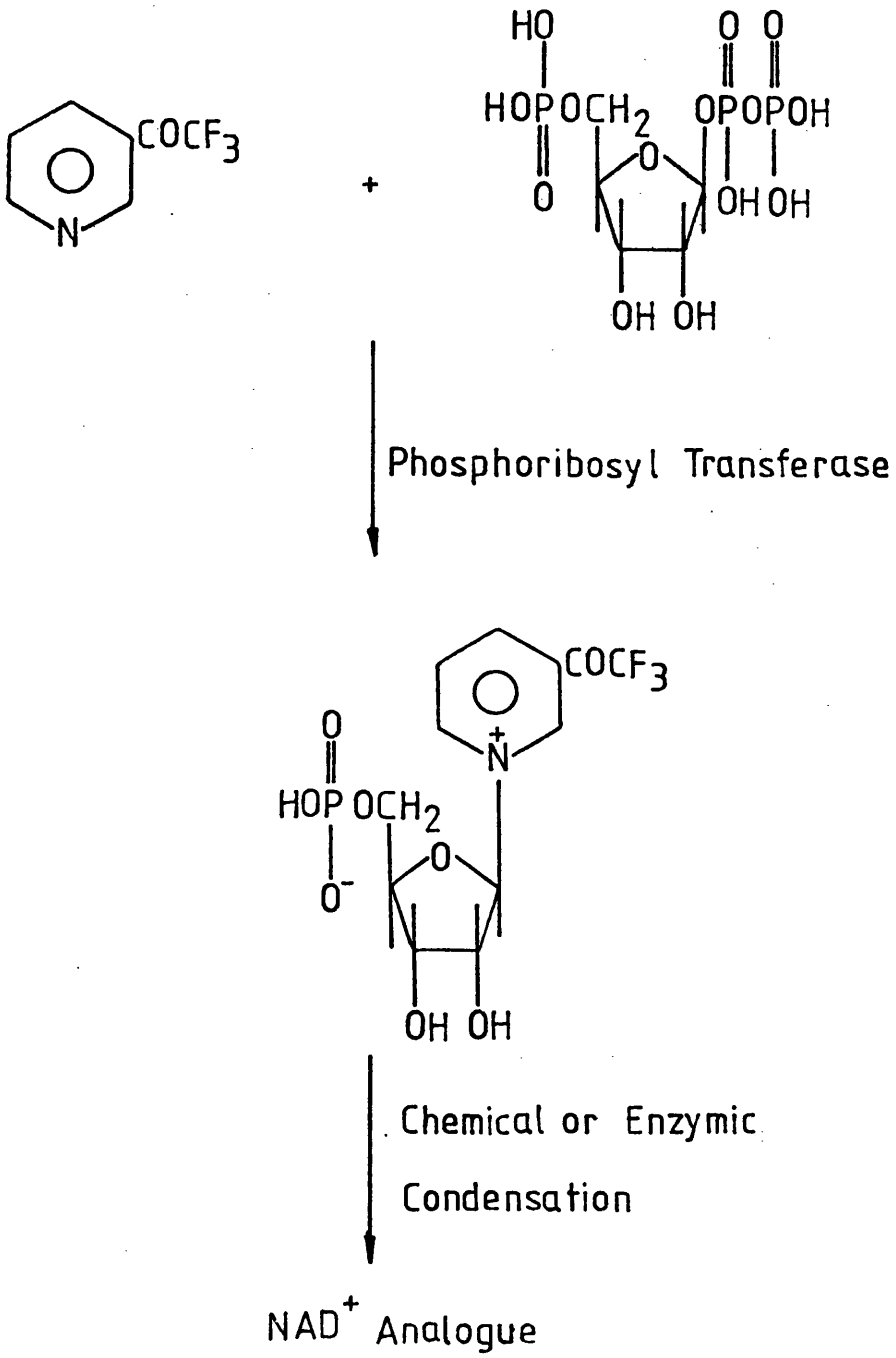
Mononucleotide preparation

An alternative method for the preparation of analogues of NAD^+ is to first prepare the nicotinamide mononucleotide analogue by using a phosphoribosyl transferase and then to join this analogue to adenosine monophosphate by either chemical or enzymic means.

The principle of this method is that the ribose-5-phosphate moiety of 5-phosphoribosyl-1-pyrophosphate (PRPP) is transferred enzymically onto the base (Fig. 47), inorganic pyrophosphate being liberated. The source of the phosphoribosyl transferase can be from either animal tissue or microorganism, and are generally specific in their base requirement.

Lactobacillus fructosus was reported by Kodama (1956) to require nicotinamide for growth and maintenance but not to use nicotinate. The biosynthetic route to NAD^+ in this microorganism was subsequently shown by Ohtsu et. al. (1967) to contain a phosphoribosyl transferase which is specific to nicotinamide and an NAD^+ pyrophosphorylase specific to NMN.

Fig. 47.



Synthesis of NAD^+ or a NAD^+ analogue using a phosphoribosyl transferase.

(3-Trifluoroacetylpyridine is used as the substituted pyridine in this example.)

The purified enzyme is stable for several months without detectable loss of activity in phosphate buffer at pH 7. The optimum activity is found to be at pH 6 to pH 7 and K_m values for nicotinamide, PRPP and ATP were found to be 2×10^{-6}M, 4×10^{-5}M and 6.7×10^{-4}M respectively.

The enzyme activity is inhibited to 20% of the original activity by a concentration of 0.1 mM of NMN, and to 60% by a similar concentration of NAD^+. These inhibitors are competitive in nature and have K_i values approximating to 7.5×10^{-4}M. Nicotinamide appears to a specific substrate for this enzyme, neither nicotinate of quinolinate phosphoribosyl transferase activity being detected in either the crude or purified enzyme preparations.

The purified enzyme catalyses the conversion of nicotinamide and PRPP to stoichemetric quantities of nicotinamide mononucleotide and pyrophosphate. The enzyme has an almost absolute specificity for ATP under the assay conditions employed.

Similar enzymes have also been isolated from animal tissues, for example, rat liver (Dietrich; 1971). It also has a dependance for ATP and nicotinamide. However, ATP is not a substrate, but acts as an allosteric modifier of NMN pyrophosphorylase. The enzyme is inhibited by nicotinamide analogues such as 6-aminonicotinamide, thionicotinamide and 3-acetylpyridine, the inhibition being competitive in nature, but not by nicotinic acid, 6-chloronicotinamide, 6-hydroxynicotinamide, aminoisonicotinamide, or ethyl nicotinate.

At least two of these nicotinamide analogues serve as substrates, thionicotinamide being utilised as well as nicotinamide, and exhibiting kinetic properties very similar to those of nicotinamide.

3-Acetylpyridine, however, has a K_m of 2×10^{-5}M, ten times that of nicotinamide, and a value of V_{max} some five times that of nicotinamide, 6-aminonicotinamide, on the other hand, inhibits the enzyme, but does not act as a substrate.

The enzyme is also inhibited by nicotinamide riboside, NMN, α-NAD^+, β-NAD^+ and several analogues of NAD^+ (Dietrich et. al.; 1968). Nicotinic acid mononucleotide, a naturally occuring analogue of NMN, has been prepared from nicotinate phosphoribosyl transferase from various sources. Preiss and Handler (1958) have for example prepared the enzyme from human erythrocytes, whilst Honjo et. al. (1966) prepared a similar enzyme from bakers yeast. The enzyme is specific for nicotinate, nicotinamide being totally inert as a substrate.

Quinolinate phosphoribosyl transferase has been isolated from various animal tissues (Iheda et. al.; 1965) and the microorganism Astasia longa (Blum and Kahn; 1971).

It is possible that one, or more of the enzymes may accept 3-trifluoroacetylpyridine as a substrate, and so catalyse the phosphoribosylation of 3-trifluoroacetylpyridine under reaction conditions similar to those employed in the various assay procedures for the enzymes mentioned.

An interesting enzyme is nicotinate ribonucleotide : benzimidazole phosphoribosyl transferase isolated from Clostridium sticklandii (Friedmann; 1965: Fyfe and Friedmann; 1969). This enzyme catalyses the exchange of ribose-5-phosphate from nicotinic acid mononucleotide to another base, in this case benzimidazole (Fig. 48).

Nicotinate mononucleotide: dimethylbenzimidazole phosphoribosyl transferase activity is also found in the enzyme preparation.

The enzyme has the disadvantage that it is relatively unstable in aqueous solution, losing 94% of its activity on storage at -15°C for 32 days. However, the stability is improved by using glycerol, ethylene glycol, or dithiothreitol during purification and storage.

The enzyme exhibits maximal activity at pH 8.6 to 9.2.

The enzyme is highly specific for a ribose-phosphate donor which contains nicotinic acid, and not nicotinamide. Hence the enzymic activity with NMN is greatly reduced, and no activity is found in the presence of NAD^+. Nicotinic acid adenine dinucleotide is less active with the enzyme than is nicotinic acid mononucleotide. Nicotinic acid ribonucleoside, nicotinamide ribonucleoside, PRPP and ribose-5-phosphate are also inactive with the enzyme and most of the activity noted with NMN is due to a deaminase which is contained in the enzyme preparation.

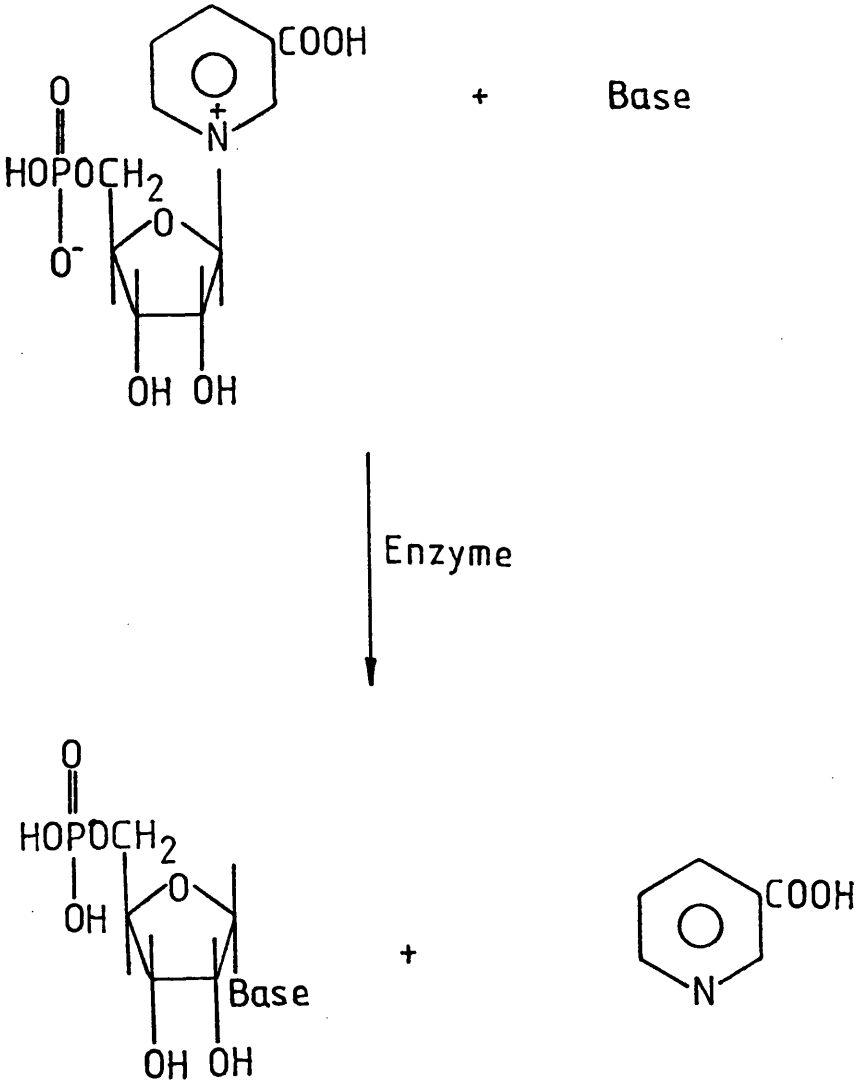
The enzyme is much less specific for the free base than it is with the ribose-phosphate donor. Benzimidazole, 5,6-dimethylbenzimidazole, 5,6-dichloro-benzimidazole and 5-nitrobenzimidazole have been shown to be active with the enzyme, the rate of the reaction being followed by means of radiotracers. The analogous enzyme from Propionibacterium shermanii has also been shown to catalyse phosphoribosyl transfer to other bases. (Friedmann; 1965).

The products obtained are the α-glycosidic-5'-ribonucleotides of the respective bases, and hence if the naturally occuring β-anomer is required the starting material would need to be the α-anomer. Owing to the lack of specificity in base requirements, this enzyme may catalyse the phosphoribosylation of 3-trifluoroacetylpyridine.

Enzymic pyrophosphorylation

Enzymic pyrophosphorylation is facilated by NAD^+ pyrophosphorylase which can again be obtained from either microorganisms or animal tissue. For example, during the preparation of nicotinamide phosphoribosyl transferase from Lactobacillus fructosus a NAD^+ pyrophosphorylase is precipitated on the addition of protamine sulphate. The enzyme may be used in the crude state or may be purified by homogenising and centrifuging, the supernatent being discarded each time. The enzyme

Fig. 48.



Action of nicotinate ribonucleotide:benzimidazole phosphoribosyl transferase.

is eluted from the pellet with 0.2M dipotassium hydrogen phosphate, the procedure giving a 10 fold increase in purity with a yield of 10%.

The enzyme is specific for NMN and does not react with nicotinic acid ribonucleotide, the final preparation being practically free of nicotinamide phosphoribosyl transferase activity. The K_m values for NMN and ATP being 6.7×10^{-4} and 2.7×10^{-3}M respectively, the maximal activity being observed at pH 5.8.

The pyrophosphorylation proceeds at pH 5.8 in tris-acetate buffer. The enzyme requires ATP and magnesium ions, the extent to which the reaction occurs being monitored by either reacting the dinucleotide with a dehydrogenase, or by incorporating a radioactive tracer into the reaction mixture, separating the components of the reaction mixture by t.l.c. and measuring the radioactivity due to each component.

Another method, based on the procedure of Kornberg (1950) utilises a pyrophosphorylase from various rat tissues. This enzyme accepts both NMN and nicotinic acid mononucleotide as substrate, although Kornberg has reported that the equalibrium constant of the reaction does not favour the formation of NAD^+.

Investigation of analogue properties.

The extent to which any fluorinated analogue can mimic the natural substrate can be examined by either classical enzyme kinetics or by ^{19}Fn.m.r. spectroscopy.

Enzyme kinetic methods.

Fluorinated analogues can act either similarly to the natural substrate (that is as an enzyme substrate) or as an inhibitor of the enzymes action on the natural substrate. The work of Eisenthal et. al. (1972) on the activity of fluoro- and deoxy- analogues of glycerol as substrates

and inhibitors of glycerol kinase provides examples of both types of analogue activity. As earlier stated (p. 16)it was shown that the analogues of glycerol in which the hydroxy groups at C-1 and C-2 were replaced by fluorine proved to be substrates, whereas that in which the C-3 hydroxy was replaced was an inhibitor.

Briley et. al. (1977 a:b) reported that difluoro-öxaloacetate behaves as a competitive inhibitor of 2-oxoglutarate and as a non-competitive inhibitor with respect to aspartate with cytoplasmic aspartate transaminase. Initially steady state kinetics were used to investigate the enzyme and rate equations for the inhibitions formulated. Subsequently kinetic analyses of both the initial fast and the overall slow reaction with the aminic form of the enzyme were made using repeated spectral scanning and stop-flow techniques, allowing the formulation of a basic reaction mechanism involving at least two intermediate enzyme complexes. A molecular reaction scheme involving a ketimine Schiffs base intermediate was also proposed.

Any technique used to investigate natural enzyme substrates can also be used with fluorinated analogues of these substrates. The above examples illustrate just some of the techniques which have been used.

^{19}F Nuclear magnetic resonance spectroscopy.

N.m.r. studies have shown that, in general, fluorine coupling constants and chemical shifts are at least an order of magnitude greater than those of the corresponding hydrogen containing compound (Emsley and Philips; 1971), while at a given magnetic field the sensitivity of the fluorine nucleus is second only to that of the proton. These facts, coupled with the minimal perterbation caused by the small fluorine nucleus when it is incorporated into a test molecule suggests that ^{19}F should act as an ideal n.m.r. probe of intermolecular interactions.

When studying enzyme systems with fluoro-compounds two approaches have

been used, firstly the fluorine may be introduced covalently onto the enzyme and the changes in the spectrum noted when an inhibitor is added. In principle this is very similar to observing the proton resonances of an enzyme in which all but a few selected protons have been deuteriated. The advantage of using a fluorine probe in these cases is that there are potentially larger changes in chemical shifts and coupling constants when the inhibitor binds.

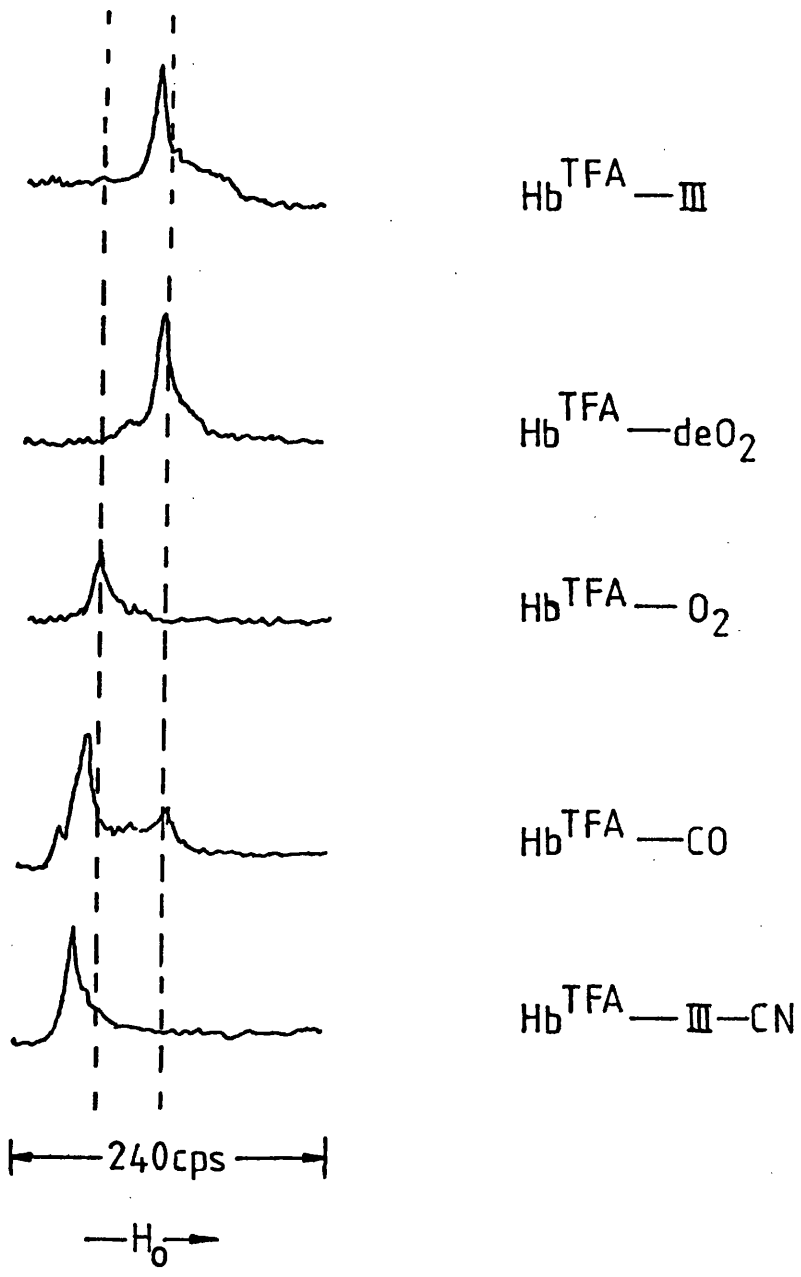
The second approach is to use a fluorinated substrate as a probe. The chemical shift, line width and other characteristics all could alter when the substrate analogue binds to the enzyme and the environment of the fluorine atom(s) changes. The relatively low concentration of bound substrate combined with the fact that, in the bound state, the signal is very broad leads to difficulties in its detection and measurement. There are, however, ways of obtaining information about the bound site. If there is fast chemical exchange between the free (in bulk) and bound substrate, then the observed signal is a weighted (time) average of that in each environment, and so carries information about the bound site.

Fluorine has been used as a covalent probe to investigate the conformational transitions in haemoglobin, which had been investigated in terms of ligand dependent induced fit (Koshland; 1953) and allosteric effects (Koshland et. al.; 1966). The use of fluorine as a probe at a specific site in hæmoglobin (Raftey et. al.; 1971) affords in principal an easier method of monitoring conformational changes than does ^{1}H n.m.r. (Wurtrich; 1971) since any changes in chemical shift will be larger than those in the corresponding proton containing compound. A 3,3,3-trifluoroacetonyl group was bound covalently and specifically to the -SH group of cysteine-β-93, thus placing the probe

close to the region of intersubunit contact (Raftery et. al.;1971). The binding of oxygen to the modified haemoglobin was essentially the same as that to natural haemoglobin. The chemical shifts of the oxy and deoxy trifluoroacetonoyl-haemoglobin complexes are shown in figure 49, together with those of the liganded forms, the small differences in chemical shift caused by the various ligands suggesting similar but not identical environments for the ^{19}F probe in each case. The pH dependance of the trifluoroacetonoyl-haemoglobin complex implicates a group with a pK_a value of 7.4 and crystallographic results (Perutz; 1970) show that the β-histidine residues at 146, 143, or 92 are possible sites. Due to the environments of the various groups this pK_a was assigned to histidine-β-146. This argument also implies that the probe will be primarily sensitive to conformational changes in the β-chain. Raftery and his coworkers also used this probe to show that, contary to previous experiments on dye binding and crystal form, methyl iron (III)-haemoglobin is different from oxy iron(III)-haemoglobin because the curve for the pH dependance of ^{19}F probe chemical shift is different. The X-ray structure does not yield precise information as the oxy-haemoglobin used in this study contained some methyl-haemoglobin. In the above example of the use of fluorine as a covalent probe precise interpretation of the chemical shift changes depends substantially on a detailed knowledge of the crystal structure. The few small enzymes for which the crystal structure is known provide a means of trying out the technique and making progress in understanding the factors which cause the chemical shifts. In larger enzymes the slower tumbling times mean that covalent probe resonances will be broader because the dipolar interactions with other nuclei will no longer be averaged out. In such cases the use of substrate probes has to be considered.

When the fluorine probe is on a substrate or an inhibitor analogue, it

Fig. 49.



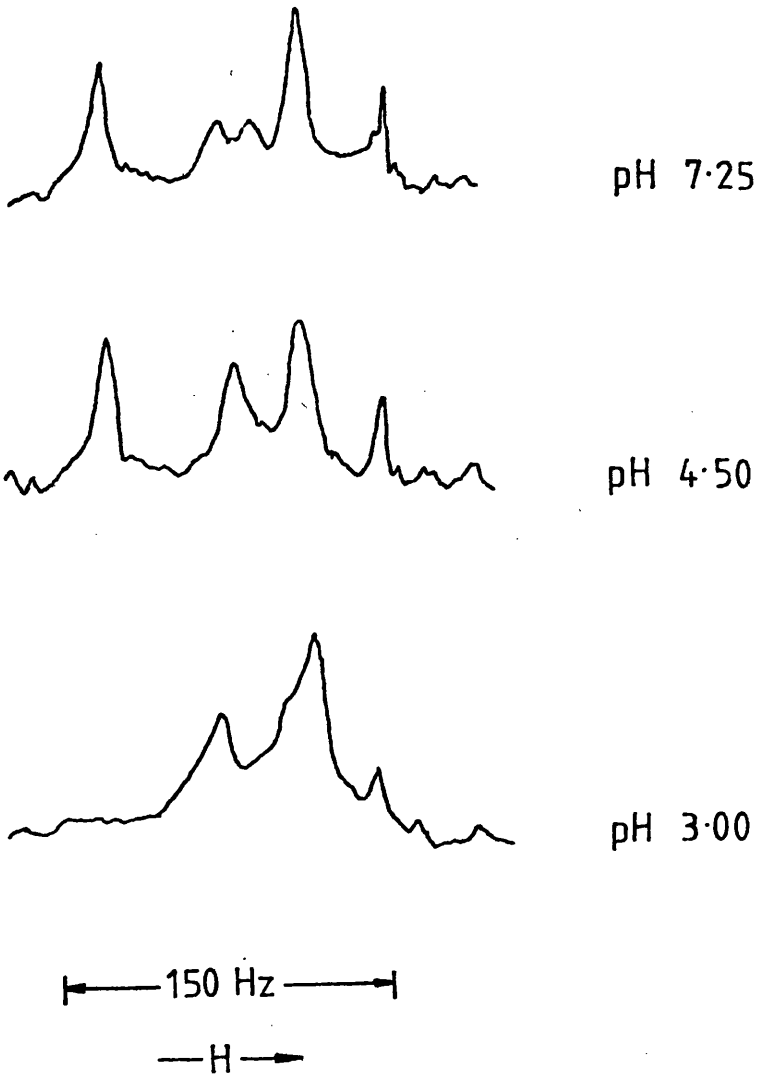
^{19}F N.m.r. spectra of trifluoroacetonylated haemoglobin with and without various ligands.

may be possible to obtain information on the fluorine resonance of the substrate or inhibitor when it is bound to the enzyme. This may result in either the observation of a weighted average resonance signal because of 'rapid chemical exchange' of the substrate between the enzyme site and the bulk, or, under conditions of 'slow chemical exchange' two signals corresponding to the bound and the free substrate will be observed.

An example when conditions of 'slow exchange' prevail is the binding of the trifluoroacetylated analogue of chitotriose to lysozyme(Raftery et. al. ; 1971). The signal of a one to one ratio of enzyme to trisaccharide (each at 3mM), at which concentration the concentration of the complex is close to 100%, is shown at 3 pH values (Fig. 50). The sharp peak at the highest field represents the trifluoroacetate anion, which is used as an internal reference. From the spectrum at pH 4.5 the resonances of the bound inhibitor were identified on the basis of experiments at high temperature, at pH 4 under conditions of 'fast exchange' with excess oligarosaccharide present, and by comparison with the spectrum of the pure trisaccharide. The effect of pH on the trifluoroacetyl group varies with the position of the group in the molecule.

The interaction of chitotriose with specific groups on the enzyme was determined by X-ray spectroscopy (Perutz ;1970). If the binding of the fluorinated analogue is similar to the natural compound the formation of a hydrogen bond between aspartate-101 and the trifluoroacetyl group would result in a change in the ^{19}F chemical shift of this group. From this model it is apparent that the trifluoroacetyl group at the reducing end of the trisaccharide is the furthest trifluoroacetyl group from aspartate-101, and yet, unlike the central trifluoroacetyl group it experiences an evident chemical shift. The effect is unlikely to be electronic, as is that for the trifluoroacetyl group at the non-reducing end of the sugar. An explanation which agrees with the

Fig. 50.



^{19}F N.m.r. spectra of solutions of 3mM-trifluoroacetylated chitotriose and 3mM-lysozyme at 3 pH values (T= 10°C). The resonance at the highest field is due to the trifluoroacetate anion.

kinetic observations using n.m.r. methods (Dahlquist and Raftery; 1972) is that the formation of the aspartate-101 hydrogen bond at the reducing end of the trisaccharide causes an isomerisation of the complex which alters the position of the end trifluoroacetate group with respect to the aromatic ring of tyrosine-108 and therefore the effect of the ring current on the fluorine nuclei.

For most inhibitors or substrates binding to enzymes conditions of 'slow exchange' do not prevail, and this is, in general, fortunate for in larger enzymes the resonances obtained would be to broad to detect.

However, under conditions of 'rapid chemical exchange' this problem does not arise. For example, it has been shown that a state of 'fast chemical exchange' exists in the binding of N-acetyl-D,L-p-fluorophenylalanine to chymotrypsin. The ^{19}F n.m.r. spectrum of the inhibitor corresponds to an AA'BB'X multiplet (Spotswood et.al. ; 1967). No difference between the ^{19}F spectra of the optical isomers was observed, but the addition of chymotripsin resulted in two sets of resonances, corresponding to the D and L isomers. Addition of the pure resolved isomers indicated that the D isomers resonance is shifted to low field, while that of the L isomer is not significantly altered.

If, under conditions of 'rapid chemical exchange', the ^{19}F n.m.r. spectrum of a substrate or inhibitor changes on binding to an enzyme it is possible to obtain the binding parameters for the system. Thus, the binding constant for the binding of N-acetyl-D-p-fluorophenylalanine (S) to chymotripsin (E)may be determined.The binding may be represented thus;-

$$E + S \rightleftharpoons ES$$

and hence:-

$$K_s = \frac{[E]\ [S]}{[ES]}$$

If the chemical shift of the fully bound substrate is represented as

Δ, and the fraction of substrate bound to the enzyme as B_f, then the observed chemical shift, δ, will be given as:-

$$\delta = B_f \cdot \Delta$$

Also, assuming that $[S_o]$ is considerably greater than $[ES]$:-

$$B_f = \frac{[ES]}{[S_o]}$$ where $[S_o]$ is the total concentration of substrate. Also:-

$$[S_o] = \frac{[E_o] \cdot \Delta}{\delta} - K_s$$

Thus, a plot of $[S_o]$ against $\frac{1}{\delta}$ allows Δ to be determined from the slope and K_s from the intercept of the x-axis. In the above example it was found that Δ =83Hz. (at 56.4KHz.) and K_s=6mM.

This equation can be modified to take into account the effects of competition by a second sustrate:-

$$\frac{1}{\delta} = \frac{1}{\Delta \cdot E_o}\left(K_s + [S_o] + \frac{K_s}{K_i} \cdot [S_i]\right)$$

where K_i and $[S_i]$ are the binding constant and total concentration of the competing substrate respectively.

It has been suggested that the low field shift observed when N-acetyl-p-fluorophenylalanine binds to chymotripsin is evidence for the binding of the aromatic ring and acetyl group of the probe into the hydrophobic 'pocket' at the active site. Although this is possibly the case there are many other factors, for example pH, temperature and through-space interactions that can contribute to ^{19}F chemical shifts, and which cannot be elucidated from a single determination of a chemical shift. The effect of pH on the chemical shift of the bound substrate may lead to further information about binding sites, if it is remembered that a detailed interpretation will depend on a knowledge of the crystal structure of the enzyme.

In their earlier investigation Briley et. al. (1977a:b) had postulated that a complex was formed between difluoro-oxaloacetate and the aldimine form of aspartate transaminase, and determined its dissociation constant spectrophotometrically and by ^{19}F n.m.r. spectroscopy. They later investigated the ^{19}F n.m.r. line width-pH and chemical shift-pH profiles of this enzyme system and found that both show inflexion points in the pH 5 and pH 8 regions and suggested that these arose from the variations in the binding of difluoro-oxaloacetate as specific groups on the enzyme are protonated (Briley et. al.; 1977c). Difluoro-oxaloacetate was also found to interact with the apoenzyme to form a complex, the dissociation constant of which was determined by ^{19}F n.m.r. spectroscopy along with the line width-pH and chemical shift-pH profiles, each of which showed a single inflexion point at approximately pH 8. The absence of the inflexion point at pH 5, which is present in the aldimine form enzyme system, indicates that this results from the ionisation of an enzyme group associated with the pyridoxal phosphate cofactor.

Compared with the magnitudes of ^{19}F chemical shifts frequently observed the changes in chemical shift in the enzyme complexes so far discussed are small. In general ^{19}F chemical shifts are large when the effects giving rise to them are transmitted through chemical bonds. For example in fluoromonosaccharides the chemical shift of the equatorial fluorine at C-3 differs by about 5 p.p.m. for the α- and β- forms. When the shifts arise from through-space interactions, such as ring currents and electric field effects, they are much smaller. In the forgoing example additions of the inhibitors or ligands may result in a change in the enzyme structure and the small shifts observed are a consequence of through-space interactions. It is possible that several of these interactions produce shifts in different direction, giving a small net chemical shift. In most experiments using enzymes, shifts will result from through-space interactions and thus, although fluorine appears

to be a highly attractive probe in enzyme systems because of the large shifts which may be expected, the smaller shifts which occur in practice are still relevent.

In larger enzymes, in which the crystal structure is unknown ^{19}F n.m.r. spectroscopy finds a great use in the mapping out of bound substrates around a paramagnetic probe, preferably one at the active site. The experiments used involve two techniques, observation of the high resolution spectrum of the substrate, and the determination of the proton relaxation behaviour of the bulk water protons. By a combination of these measurements it is possible in principle to calculate the distances of individual nuclei from the paramagnetic probe. The relaxation times are related to this distance by the Solomon-Bloemberger equations. The calculation of the distances requires a knowledge of the correlation time; a parameter difficult to determine without detailed relaxation time measurements as a function of magnetic field and temperature. However, the use of proton relaxation enhancement measurements suggests a fairly straightforward way of obtaining reasonable estimates of correlation time(Dwek; 1972).

The study of the proton relaxation of the water molecules around the paramagnetic ion in the presence of different amounts of macromolecule or ligand allows the determination of the stability constant for the paramagnetic ion-linked binary complexes. The addition of substrate to form the metal ion-macromolecule-substrate complex gives ternary complexes, whereas the addition of further substrate will form a quarternary complex.

In addition to allowing the determination of binding constants the binary, ternary and quarternary enhancement parameters of the fully formed complexes may be obtained and these are believed to be characteristic for a given state or conformation of an enzyme.

Summarising, ^{19}F relaxation times are increased by the addition of paramagnetic metal ions, which have relatively long electron spin lattice relaxation times. The increase in the relaxation times is a function of the distance between the metal ion and the observed nucleus and of the correlation times for the n.m.r. relaxation behaviour of the complex. The introduction of metal ions such as Mn (II) and Gd (III) into enzyme systems thus allows molecular conformation determinations of inhibitor-enzyme complexes with respect to the metal site.

An example of the use of paramagnetic metal ion is the binding of methyl N-fluoroacetyl-β-D-glucosamine, and of N-fluoroacetylglucosamine to lysozyme. The addition of lysozyme to the methyl compound (Butchard et.al.; 1972) results in a downfield chemical shift, which can be used to determine the binding constant and chemical shift of the bound substrate by means similar to those shown above(p 57). The paramagnetic probe chosen was Gd (III), since X-ray crystallography had shown this ion to bind at the active site. Also the chemistry of the lanthanides parallels that of the actinides, so that if the position of a heavy metal atom is known from X-ray work it is likely that the corresponding lanthanide ion (Gd (III) in this case) binds at the same site.

The addition of Gd (III) results in a broadening of the resonance shown by the pure substrate and a much greater broadening when the enzyme is present. The broadening for the pure substrate probably arises from dipolar interactions, or the formation of a very weak complex. The coordination number and dissociation constant were determined from the proton relaxation enhancement experiments, as well as the internuclei distances.

Similar experiments on the binding of N-fluoroacetylglucosamine to lysozyme also enabled the internuclei distances to be found for both

the α- and β- anomers and allowed the postulation that the chemical shift of the β- anomer is the result of the influence of tryptophen-108, whereas that of the α- anomer is due to the influence of tryptophen-63 in lysozyme.

This example illustrates that with the use of distance information from line broadening studies, complemented by the geometric information obtained from chemical shift studies caused by through-space, dipolar interactions it is possible to accurately locate the position of the nuclei studied.

From the above examples it can be seen that fluorinated analogues can, by using ^{19}F n.m.r. spectroscopy, yield a considerable amount of information about enzymes and the way in which they act. In view of the central role played by NAD^+ in biochemical processes it was considered that a fluorinated analogue of this compound would prove of wide use as an enzyme and metabolic probe.

EXPERIMENTAL

and

RESULTS

MATERIALS

All chemicals, except where stated in the text, were obtained from British Drug Houses, and were, where available, of 'AnalaR' quality. Solvents were, where necessary, dried over sodium wire, except that (a) alcohols were dried by refluxing them with magnesium turnings and iodine, followed by distillation and (b) chloroform and the toluene/dioxan mixture (used in perchloryl fluoride fluorinations) were dried over molecular sieve. Enzymes and other biochemicals were, except where stated in the text, obtained from the Boehringer Corporation. Radiochemicals were obtained from the Radiochemical Centre.

INSTRUMENTATION

Infra-red spectra were measured (over the range 4000 to 670 cm^{-1}.) using a Perkin-Elmer 137 spectrophotometer.

Gas liquid chromatography was performed on columns (2m x 6mm) of coiled stainless steel, packed with 3% silicone gum-rubber (SE-301) on High performance Chromosorb W (80 to 100 mesh). Samples were chromatographed isothermally, at 100°C, using a Perkin-Elmer F11 gas chromatograph equipped with a flame ionisation detector. Concentrations were calculated on the basis of peak area.

Ultra-violet/visible spectra and enzyme kinetic measurements were recorded using a Pye-Unicam SP1800 spectrophotometer using 1 cm silica cells.

Nuclear magnetic resonance spectra were recorded on a Jeol PS-100 n.m.r. spectrometer, and where necessary the signal to noise ratio was enhanced by means of repeated scans using a Jeol JNB SB-3 signal-to-noise booster. Proton n.m.r. chemical shifts are quoted in p.p.m. downfield from tetramethylsilane, whereas ^{19}F n.m.r. chemical shifts are given in p.p.m. from trifluoroacetic acid.

Mass spectra were recorded using an A.E.I. M.S. 12 spectrophotometer (at 70 eV.), and are quoted in graphical form.

DETECTION OF FLUORINE

The compound under investigation (ca. 50mg) was fused with sodium (0.2 g) and the reaction mixture was quenched in distilled water (5 ml). The inorganic salts were dissolved by boiling with glacial acetic acid (0.5 ml) and the solution concentrated to low volume (ca. 1 ml). A few drops of this solution were added to 2 ml of a 50% mixture of alizarin red S (saturated in ethanol) and zirconoyl chloride (5% in 50% HCl). A change in colour from red to yellow indicated the presence of fluorine.

DETECTION OF CHLORIDE

A few drops of a solution of the compound under investigation in 5% nitric acidwere added to an aqueous solution of silver nitrate. A white precipitate indicated the presence of chloride.

DETECTION OF CARBOHYDRATE

The Molisch test was used to detect carbohydrates. The compound under test (5 mg) was dissolved in water (0.5 ml), and 2 drops of 1-naphthol solution (10% in ethanol) were added. Concentrated sulphuric acid (1 ml) was added carefully, a red ring at the interface between the layers indicated the presence of a carbohydrate.

CHEMICAL SYNTHESES OF PRIMARY INTERMEDIATES

Ethyl nicotinate hydrochloride.

Ethyl nicotinate (0.33 moles)was dissolved in dry diethyl ether (200 ml) and dry HCl was passed through the solution. The crystals which formed were recovered by filtration and purified by recrystallisation from ethanol:diethyl ether (1:1).

The product was isolated as white needle crystals in 95% yield, and had the following spectroscopic data:-

I R spectrum.(Nujol mull)

Major absorptions:- 3040, 1740 (ester $\rangle C{=}O$), 1620, 1600, 1525, 1450,

and 1320cm^{-1}.

N.m.r. spectrum (in deuterium oxide).

1.47p.p.m. (triplet, 3 protons, CH_3 of the ethyl group, J = 7Hz.),

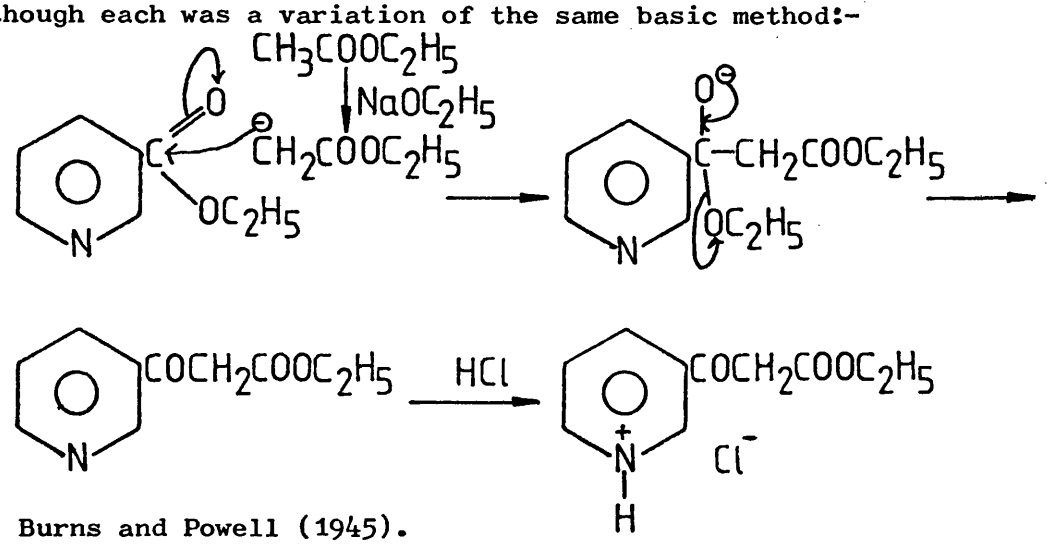
4.54p.p.m. (quartet, 2 protons, CH_2 of the ethyl group, J = 7Hz.),

and 8.22 to 9.42p.p.m. (multiplet, 4 protons, protons on the pyridine ring).

The above data confirms that the product was ethyl nicotinate hydrochloride.

Ethyl nicotinoylacetate hydrochloride.

Three methods for the synthesis of this compound were examined, although each was a variation of the same basic method:-



(a) Burns and Powell (1945).

The product was obtained as pale yellow crystals (m.p. 152 to 154°C) in 66% yield. Burns and Powell quote a 37% yield of cream crystals (m.p. 140°C (decomposition), then melting at 154 to 155°C).

(b) Strong and McElvain (1933).

The product was obtained as pale yellow crystals (m.p. 155 to 156°C) in 75% yield. Strong and McElvain quote a 70% yield of cream crystals (m.p. 156 to 157.5°C).

(c) Gilman et. al. (1948).

The above method was followed, except that the product was isolated

as the hydrochloride, whereas Gilman isolated ethyl nicotinoylacetate itself. The product was isolated as yellow crystals (m.p. 152 to 157°C) in 29% yield. Gilman isolated ethyl nicotinoylacetate as a straw coloured liquid (b.p. 121 to 123°C(0.4 mm Hg)) in 67% yield. The spectroscopic properties of the product obtained from each of the three preparations were similar, and showed that the product was the desired compound, ethyl nicotinoylacetate hydrochloride.

I.R. spectrum (Nujol mull).

Major absorptions:- 3050 (aromatic $\geq$C—H), 1740 (ester $\rangle$C=O), 1695 (ketonic $\rangle$C=O), 1635, 1600, 1530, 1455 and 1335 cm^{-1}.

N.m.r. spectrum (in D^6-DMSO).

1.16p.p.m. (triplet, 3 protons, CH_3 of the ethyl group, J = 7Hz.), 4.06p.p.m. (quartet, 2 protons, CH_2 of the ethyl group, J = 7Hz.), 4.35p.p.m.* (singlet, 2 protons, methylenic $-CH_2-$ group), 7.96 to 9.37p.p.m. (multiplet, 4 protons, protons on the pyridine ring) and 9.51p.p.m.* (singlet, 1 proton , $\geq$N—H).

* These resonances disappeared on the addition of D_2O.

N.m.r. spectrum (in deuterium oxide).

1.27p.p.m. (triplet, 3 protons, CH_3 of the ethyl group, J = 8Hz.), 4.27p.p.m. (quartet, 2 protons, CH_2 of the ethyl group, J = 8Hz.) and 8.20 to 9.40p.p.m. (multiplet, 4 protons, protons on the pyridine ring).

No resonances due to ethyl nicotinate hydrochloride were found.

Mass spectrum.

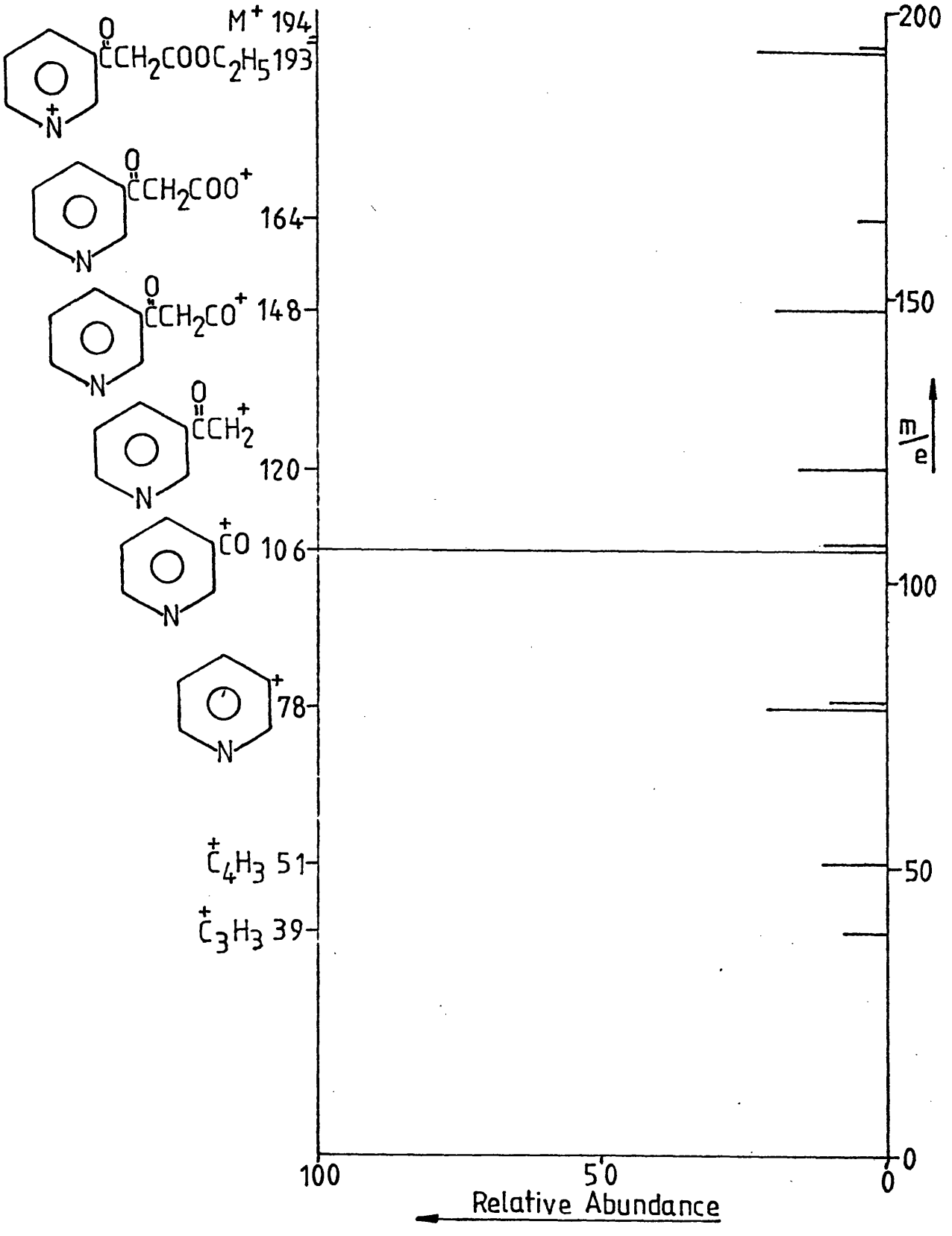
The interpreted mass spectrum is shown in figure 51.

Ethyl nicotinoylacetate.

Ethyl nicotinoylacetate hydrochloride (0.05 moles) was dissolved in water (50 ml), diethyl ether (200 ml) was added and the aqueous layer was neutralised with Na_2CO_3. The ether layer was separated and the aqueous

Fig. 51.

Mass spectrum of ethyl nicotinoylacetate hydrochloride.



layer was extracted with further ether (2 x 50 ml). The ether layer and the extracts were combined and dried ($MgSO_4$) and the ether was removed in vacuo (at ca. 30°C /300 mm Hg) to give a pale yellow viscous liquid (63% yield).

The product was distilled under reduced pressure at 33 Nm^{-2}(0.25 mm Hg) to give four fractions and a residue. The major fraction (89% of the distillation input) had a boiling point of 115°C /33 Nm^{-2} (0.25 mmHg) and the spectroscopic data was consistent with the product having the desired structure.

I.R. spectrum (Liquid film).

Major absorptions:- 2960 (≥C—H stretch), 1740 (ester >C==O), 1695 (ketonic >C==O),1650, 1625, 1585, 1420, 1320, 1265, 1210, 1020, 803 and 704 cm^{-1}.

N.m.r. spectrum (in $CDCl_3$).

1.20 p.p.m. (triplet, 3 protons, CH_3 of the ethyl group, J = 8 Hz.), 3.99 p.p.m. (quartet, 2 protons, CH_2 of the ethyl group, J = 8 Hz.), 4.40 p.p.m.* (singlet, 2 protons, methylenic $-CH_2-$ group) and 7.30 to 8.62 p.p.m. (multiplet, 4 protons, protons on the pyridine ring).

* This resonance disappeared on the addition of D_2O.

Mass spectrum.

The interpreted mass spectrum is shown in figure 52.

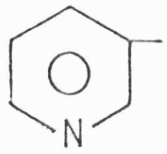
Gas liquid chromatography.

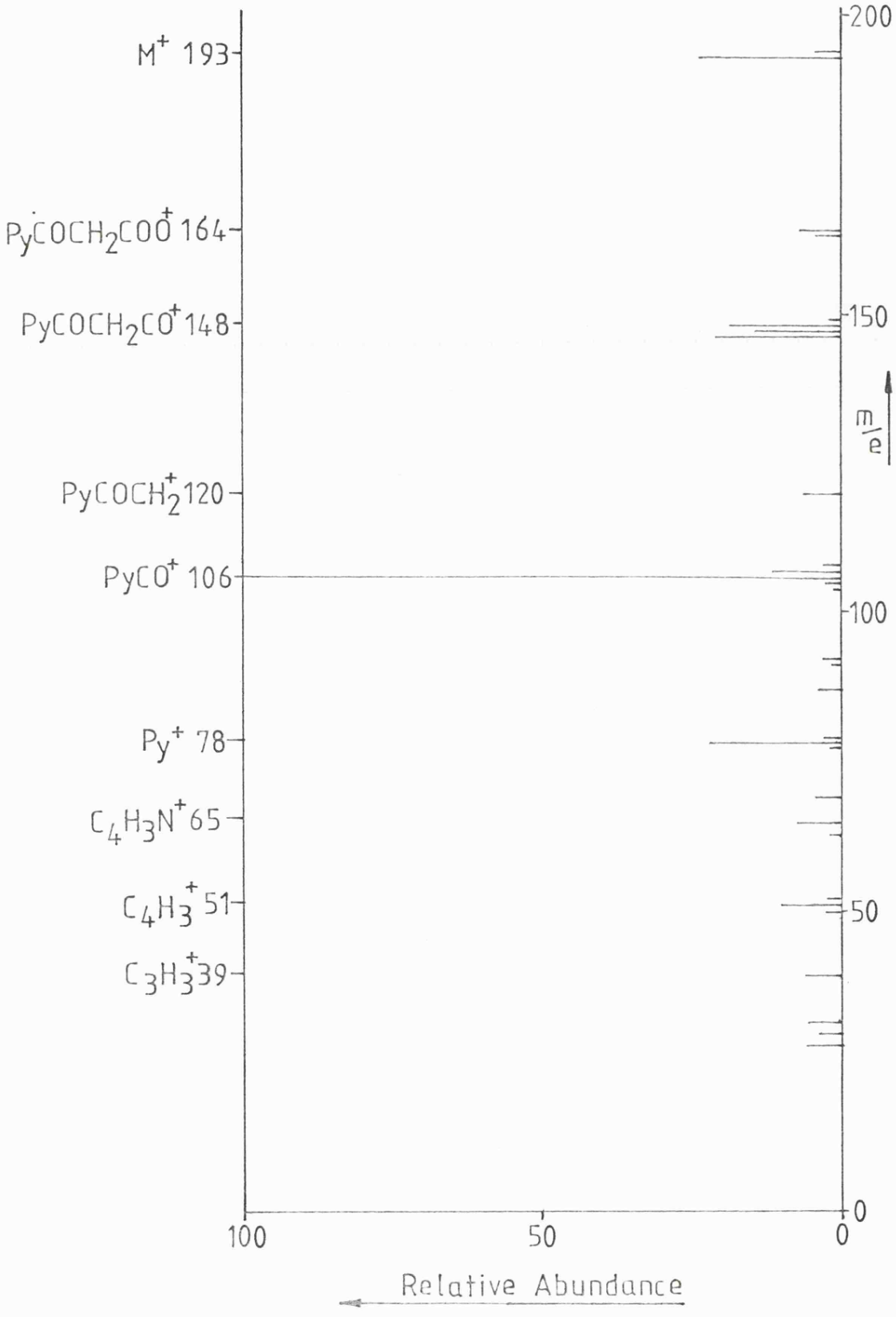
Several compounds were eluted. The two major components had retention times of 2.51 minutes (3%, ethyl nicotinate) and 12.42 minutes (95%, ethyl nicotinoylacetate).

Fractions from the vacuum distillation which had a lower boiling point than the main fraction were shown, by I.R. spectroscopy, to contain a mixture of the product, ethyl nicotinoylacetate, and the starting material, ethyl nicotinate. The residue was shown to contain only a little product.

Fig. 52.

Mass spectrum of ethyl nicotinoylacetate.

Py ≡ 



Ethyl difluoronicotinoylacetate.

The fluorination method used by Briley et. al. (1977a) to prepare diethyl difluorooxaloacetate from diethyl oxaloacetate was used in the preparation of the above compound.

Ethyl nicotinoylacetate (0.05 moles) in dry toluene:dioxan (2:1) was stirred under an atmosphere of dry nitrogen. The mixture was cooled to -5°C (the temperature of the reaction mixture was kept at between 0 and -5°C throughout the fluorination) and dry perchloryl fluoride (Pennsalt Chemical Corporation) was passed through the reaction mixture at a flow rate of 1 l hr^{-1} for 30 minutes. A suspension of sodium hydride (0.12 moles, obtained as a 50% dispersion in mineral oil from The Aldrich Chemical Company) in dry toluene:dioxan (2:1) (15 ml) was added to the reaction mixture over a period of one hour, during which time the flow of perchloryl fluoride was continued. Gas was evolved, and the solution became yellow.

The flow of perchloryl fluoride was continued, and the extent of the reaction was monitored by analysis of samples using ultra-violet spectroscopy, the starting material having a λ_{max} at 288 nm and the product at 277 nm. When the reaction appeared to be complete the flow of perchloryl fluoride was stopped and the residual gas was flushed from the system by dry nitrogen. Water (10 ml) was added to the reaction mixture in order to decompose the excess sodium hydride and to dissolve the inorganic salts, followed by saturated NaCl (100 ml) and diethyl ether (250 ml). The organic layer was separated and successively washed with water (200 ml) and saturated aqueous NaCl (100 ml). The washings were retained. The aqueous layer was sequencially washed with diethyl ether (3 x 250 ml) and benzene (2 x 250 ml). Each of the extracts was washed with the water and the saturated NaCl retained from washing the original organic layer. The organic layers were combined,

dried ($MgSO_4$) and the solvents removed in vacuo (at ca. 30°C /400 mm Hg) to give two layers, one, a colourless layer, presumably being derived from the NaH /mineral oil dispersion, and the other, a dark brown, viscous liquid, possibly being the product.

The brown layer (48% yield) was distilled under reduced pressure at $20Nm^{-2}$ (0.15 mm Hg) yielding three fractions and a residue. The main fraction (84% of the distillation input; b.p. 95 to 98°C /20 Nm^{-2} (0.15 mm Hg)) was shown to be mainly the required product, ethyl difluoronicotinoylacetate.

I.R. spectrum (Liquid film).

Major absorptions:- 2960 (>C—H stretch), 1770 (ester >C=O), 1705 (ketonic >C=O), 1580, 1415, 1310, 1260, 1160 (>C—F), 1122 (>C—F), 922 and 700 cm^{-1}.

N.m.r. spectrum (In $CDCl_3$).

^1H :- 1.23 p.p.m. (triplet, 3 protons, CH_3 of the ethyl group, J = 10 Hz.), 4.02 p.p.m. (quartet, 2 protons, CH_2 of the ethyl group, J = 10 Hz.), 4.35 p.p.m. (doublet, 0.15 protons, >C—H of the -CHF- group of the monofluoro compound ?, J = 56 Hz.), 4.40 p.p.m. (singlet, 0.2 protons, methylenic $-CH_2-$ group from ethyl nicotinoylacetate), 7.90 to 8.70 p.p.m. (multiplet, 4.1 protons, protons on the pyridine ring).

^{19}F :- 7.1 p.p.m. downfield (singlet, 1 fluorine, CF_2 group from the desired product) and 5.43 p.p.m. downfield (doublet, 0.05 fluorines, >C—F of the -CHF- group in the monofluoro compound ?, J = 56 Hz.)

Mass spectrum.

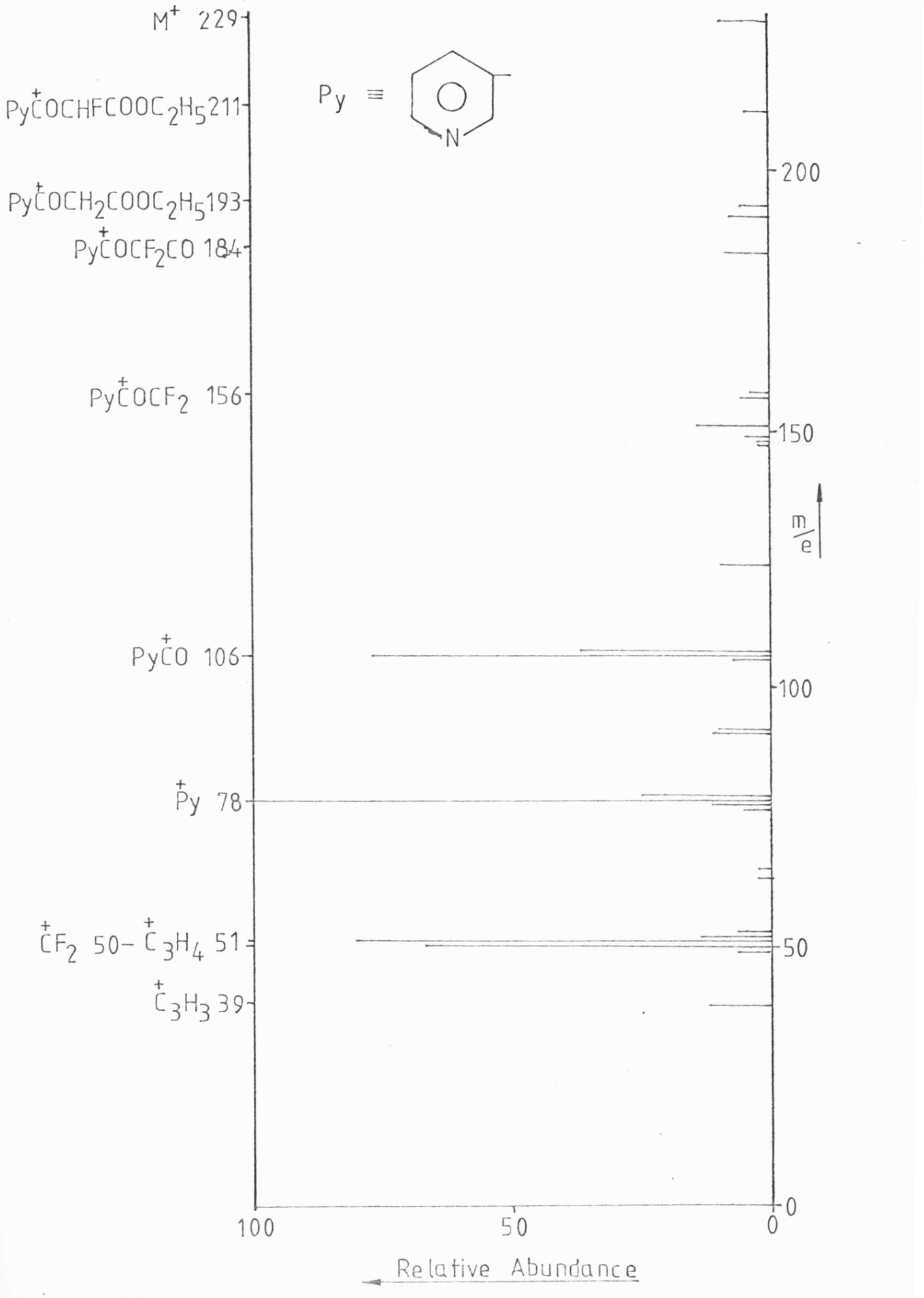
The interpreted mass spectrum is shown in figure 53.

Gas liquid chromatography.

Five compounds were eluted. These had retention times of 2.50 minutes (1%, ethyl nicotinate), 3.09 minutes (1%, unknown), 6.19 minutes (80%, ethyl difluoronicotinoylacetate), 9.59 minutes (10%, ethyl

Fig. 53.

Mass spectrum of ethyl difluoronicotinoylacetate.



fluoronicotinoylacetate ?) and 12.40 minutes (8%, ethyl nicotinoylacetate).

Fractions with a higher boiling point were shown, by I.R. spectroscopy, to contain a mixture of ethyl difluoronicotinoylacetate and the starting material. The residue was hydrocarbon in nature.

The results quoted above represent the best yield of ethyl difluoronicotinoylacetate which was obtained. Subsequent fluorinations, which were monitored by means of g.l.c. rather than ultra-violet spectroscopy, gave very little ethyl difluoronicotinoylacetate, the reaction only proceeding to give ethyl fluoronicotinoylacetate in most cases. Catalytic amounts (0.1 ml) of water or ethanol, as well as additional sodium hydride, were added to the reaction mixture in an attempt to obtain a greater yield of ethyl difluoronicotinoylacetate. n-Butyllithium (Gilman et. al.; 1949) was used as the base in place of sodium hydride in one preparation, but the isolated product appeared, by I.R. spectroscopy, to contain mainly ethyl nicotinoylacetate, the starting material.

Difluoronicotinoylacetic acid hydrochloride.

Ethyl difluoronicotinoylacetate (0.013 moles) and 25% HCl (40 ml) were heated under reflux for 4 hours, the solution was reduced to low volume in vacuo, and the residual water was removed as the benzene azeotrope. The residue was recrystallised from diethyl ether:ethanol (1:1) to give yellow crystals of difluoronicotinoylacetic acid hydrochloride (32% yield).

I.R. spectrum (Nujol mull).

Major absorptions:- 3300 (hydrogen bonded -OH), 1715 (ketonic $\rangle C{=}{=}O$), 1685 (carboxylic acid $\rangle C{=}{=}O$), 1600, 1560 and 1090 cm^{-1} ($\rangle C{-}F$).

The above spectroscopic data suggests that difluoronicotinoylacetic acid hydrochloride was formed.

3-Difluoroacetylpyridine.

Difluoronicotinoylacetic acid hydrochloride (0.002 moles) was dissolved in water (10 ml), the pH of the solution was adjusted to 7.5 with Na_2CO_3 and 1N-NaOH (2 ml) was added. The mixture was heated under reflux for $2\frac{1}{2}$ hours, cooled, and the solution was extracted with diethyl ether (4 x 10 ml). The extracts were dried ($MgSO_4$) and the ether was removed in vacuo to give a pale yellow powder (m.p. 30 to 32°C) which was thought to be 3-difluoronicotinoylacetic acid (yield 8%).

I.R. spectrum (Liquid film).

Major absorptions:- 3300 (hydrogen bonded -OH), 1710 (ketonic $\rangle C{=}O$), 1695 (carboxylic acid $\rangle C{=}O$),1590, 1560, 1485 and 1185 cm^{-1} ($\rangle C{-}F$).

The above spectrum is inconsistent with the product obtained being 3-difluoroacetylpyridine. The most likely structure for the product is 3-difluoronicotinoylacetic acid. However, the spectroscopic data obtained for this compound and for difluoronicotinoylacetic acid hydrochloride was insufficient to make a positive identification of these substances.

N,N-Diethyltrifluoroacetamide.

This compound was prepared by the reaction of diethylamine and trifluoroacetyl chloride (Henne et. al.;1948) according to the method of Zaitseva et. al. (1961). The product was purified by distillation under reduced pressure to give a major fraction (b.p. 84 to 85°C / 6650 Nm^{-2} (50 mm Hg))in 52% yield, the properties of which were consistent with its identification as the desired product, N,N-diethyltrifluoroacetamide. Zaitseva quotes a 48% yield of a colourless liquid (b.p. 74 to 75°C /50 mm Hg).

I.R. spectrum (Liquid film).

Major absorptions:- 2990 ($\rangle C{-}H$ stretch), 1685 (amide $\rangle C{=}O$),1460,

a group of five peaks from 1230 to 1120 ($\rangle C—F$), 878 and 760 cm^{-1}.

N.m.r. spectrum (in $CDCl_3$).

^1H :- 1.23 p.p.m. (triplet, 3 protons, CH_3 of one of the ethyl groups, J = 7.2 Hz.), 1.29 p.p.m. (triplet, 3 protons, CH_3 of the other ethyl group, J = 7.2 Hz.) and 3.60 p.p.m. (quartet, 4 protons, CH_2 of both ethyl groups, J = 7.2 Hz.).

^{19}F :- 8.94 p.p.m. downfield (singlet, CF_3 group).

Mass spectrum.

The interpreted mass spectrum is shown in figure 54.

Other fractions from the distillation were shown, by I.R. spectroscopy, to contain no significant amount of the desired product.

3-Trifluoroacetylpyridine.

3-Bromopyridine (Aldrich Chemical Company) and n-butyllithium (Gilman et. al.; 1949) were reacted to form 3-lithiopyridine (Wibaut et. al.; 1951) and the product from this reaction was reacted with N,N-diethyltrifluoroacetamide according to the method of Salvador and Saucier (1971) to give a dark brown viscous liquid (80% yield). The product was purified by distillation under reduced pressure, when a main fraction (75% of the input to the distillation, b.p. 71 to 73°C/ 5320 Nm^{-2} (41 mm Hg)) was obtained, the properties of which were consistent with those reported and showed the product to be the desired compound, 3-trifluoroacetylpyridine.

Salvador and Saucier quote a boiling point of 98 to 100°C /68 mm Hg and a frequency for the carbonyl absorption (CCl_4 solution) of 1725 cm^{-1}.

I.R. spectrum(CCl_4 solution).

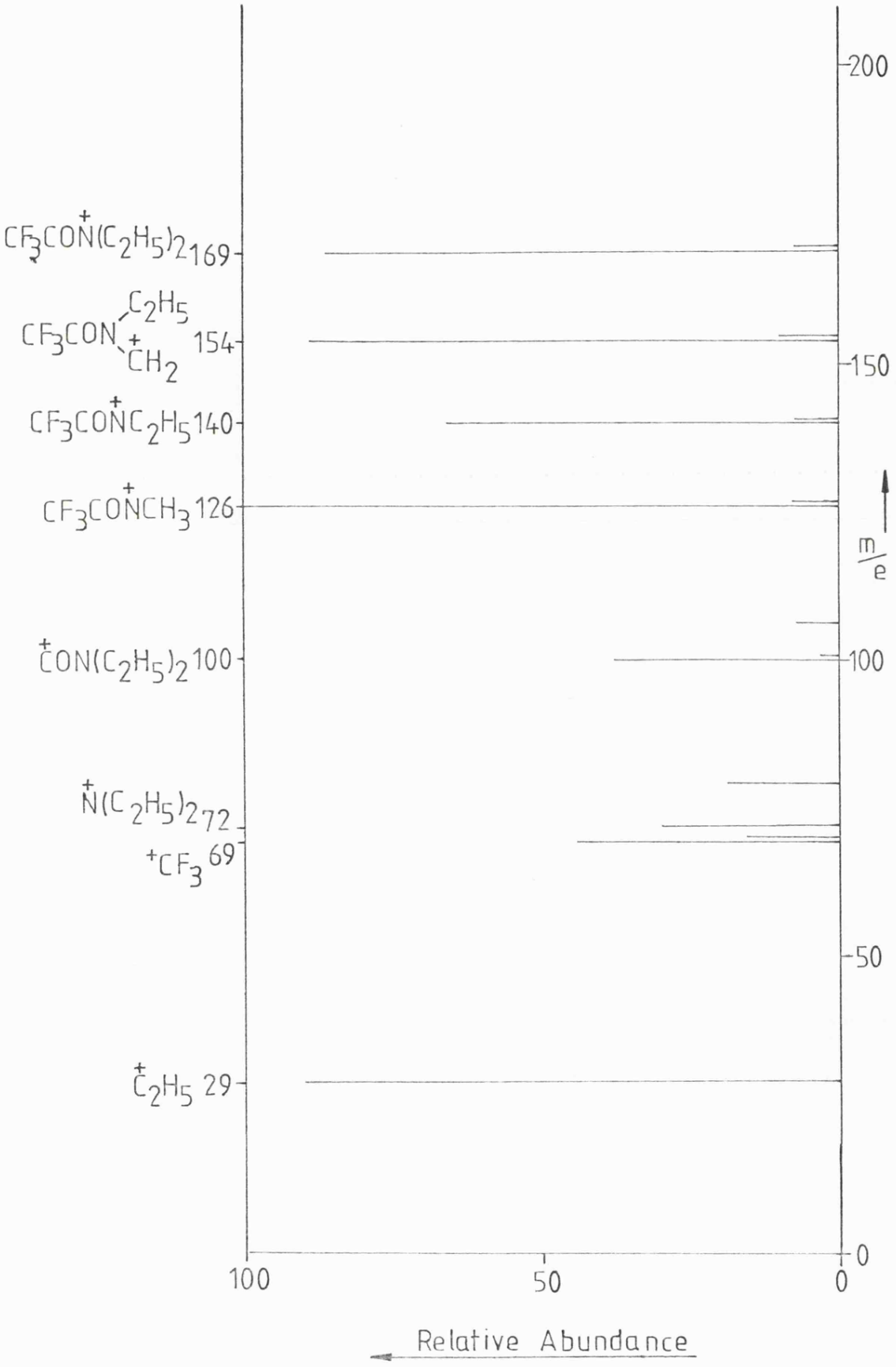
$\rangle C{=}{=}O$ absorption at 1723 cm^{-1}.

I.R. spectrum (Liquid film).

Major absorptions:- 1720 (ketonic $\rangle C{=}{=}O$), 1590, 1475, 1415, 1205

Fig. 54.

Mass spectrum of N,N-diethyltrifluoroacetamide.



(>C—F), 1175 (>C—F), 1143 (>C—F), 940 and 695 cm^{-1}.

The three peaks at 1205, 1175, and 1143 cm^{-1} are at similar frequencies to peaks found in the spectra of other compounds containing a trifluoroacetyl group, and may be attributed to this grouping (Aldrich Libary of Infra-red Spectra).

N.m.r. spectrum (in D^6-acetone).

^1H:- 7.2 to 9.1 p.p.m. (multiplet, protons on the pyridine ring).

^{19}F:- 6.36 p.p.m. upfield (singlet, CF_3 of the trifluoroacetyl group).

Mass spectrum.

The interpreted mass spectrum is shown in figure 55.

Gas liquid chromatography.

Two compounds were eluted. These had retention times of 6.92 minutes (93.5%, 3-trifluoroacetylpyridine) and 10.08 minutes (6.5%, 3-bromopyridine). In subsequent preparations the purity of the product was improved to 98.1%.

3-Trifluoroacetylpyridine gem-diol.

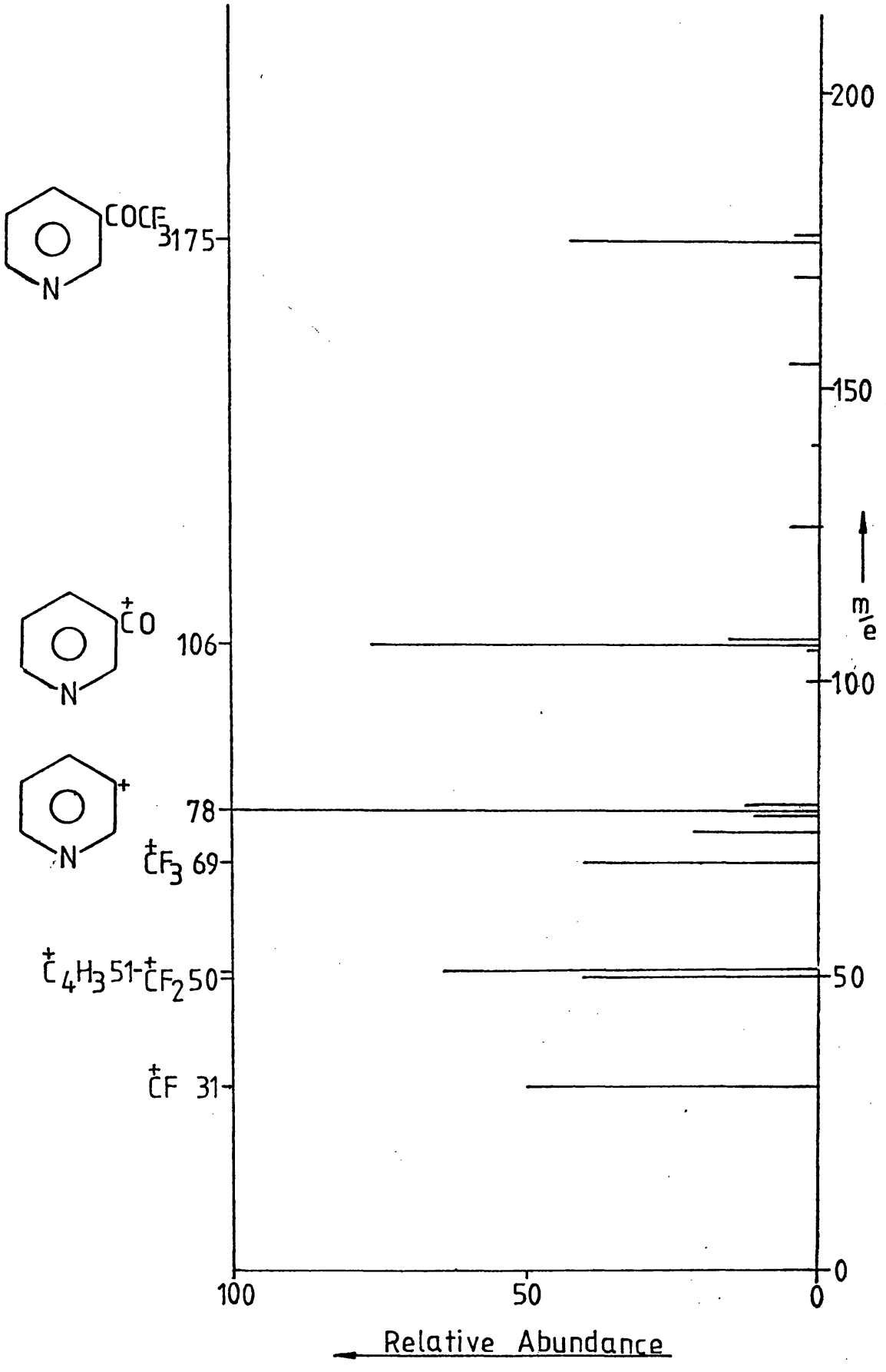
This compound was prepared by leaving 3-trifluoroacetylpyridine in a moist atmosphere overnight, according to the method of Salvador and Saucier (1971). 3-Trifluoroacetylpyridine gem-diol was obtained as white crystals (m.p. 92 to 93°C) in 95% yield. Salvador and Saucier quote a yield of 69.4% (from 3-bromopyridine)and a melting point of 93 to 96°C. They also quote that the I.R. spectrum contains a broad -OH absorption at 3480 cm^{-1} and that in the n.m.r. spectrum the protons of the -OH groups have a chemical shift of 7.38p.p.m.. The product had the following spectroscopic data, which is consistent with that quoted by Salvador and Saucier:-

I.R. spectrum (Nujol mull).

Major absorptions:- 3450 (hydrogen bonded -OH), 1600, 1460, 1180 (>C—F), 1070 (>C—F), 930, 813 and 710 cm^{-1}.

Fig. 55.

Mass spectrum of 3-trifluoroacetylpyridine.



N.m.r. spectrum (in tetrahydrofuran).

^{1}H:- 7.3 to 9.0 p.p.m. (multiplet, 4 protons, protons on the pyridine ring) and 7.40 p.p.m.* (singlet, 2 protons, protons of the -OH groups).

* This resonance disappeared when D_2O was added.

^{19}F:- 6.30 p.p.m. upfield (singlet, CF_3 group).

3-Trifluoroacetylpyridine-2,4-dinitrophenylhydrazone.

3-Trifluoroacetylpyridine (0.001 moles) was added to a solution of 2,4-dinitrophenylhydrazine (0.5 g) in methanol (5 ml)and sulphuric acid (0.5 ml). The solid which formed was separated by filtration, washed with aqueous methanol, then with dilute sodium hydrogen carbonate solution and recrystallised from aqueous methanol (75%) to give red crystals in 75% yield.

Ultra-violet spectrum.

$\lambda_{max\ (CHCl_3)} = 375$ nm ($\varepsilon = 3.01 \times 10^4\ cm^2M^{-1}$)

3-Diazoacetylpyridine.

This compound was prepared by the method of Dornow (1940) from nicotinoyl chloride and diazomethane. 3-Diazoacetylpyridine was obtained as colourless prisms (m.p. 72 to 73°C)in 19% yield. Dornow quotes a 20% yield and a melting point of 74°C.

o-Phenylene phosphorochloridate.

Phosphorus pentachloride and o-dihydroxybenzene were reacted to form 2,2,2-trichloro-1,3,2-benzodioxaphosph(V)ole which was then reacted with acetic anhydride according to the method of Khwaja et. al. (1970) to give the above compound,which was isolated as yellow crystals (m.p. 56 to 58°C; b.p. 130 to 131°C /2660 Nm^{-2} (20 mm Hg)) with a yield of 42%. Khwaja quotes a melting point of 58 to 59°C, a boiling point of 122°C (12 mm Hg) and a yield of 65%.

1-O-Acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose.

The above compound was prepared by one series of operations involving the methylation, benzoylation, demethylation and acetylation of D-ribose according to the method of Kissmann et. al. (1955). The product was isolated as white crystals (m.p. 127 to 128°C) in 79% yield. Kissmann et. al. quote a 57% yield of white crystals which had a melting point of 126 to 129°C. The product had the following spectroscopic data which was consistent with its being the desired product, 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose.

I.R. spectrum (Nujol mull).

Major absorptions:- 1745 (ester $\rangle C{=}{=}O$ of the acetyl group), 1725 (ester $\rangle C{=}{=}O$ of the benzoyl groups), 1450, 1270 ($\rangle C{-}O$ stretch of the benzoyl ester groups), 1225 ($\rangle C{-}O$ stretch of the acetyl ester group), 1120, 1022, 970, 952 and 701 cm^{-1}.

N.m.r. spectrum (in $CDCl_3$).

The protons on the ribofuranose ring are given alphabetic assignments in figure 56. These assignments are used in the following table.

2.02 p.p.m. (singlet, 3 protons, CH_3 of the acetyl group), 4.42 to 4.92 p.p.m. (multiplet, 3 protons, ribofuranosyl protons 'a', 'b' and 'c'), 5.79 to 6.01 p.p.m. (multiplet, 2 protons, ribofuranosyl protons 'd' and 'f'), 6.47 p.p.m. (singlet, 1 proton, ribofuranosyl proton 'e'), 7.28 to 7.68 p.p.m. (multiplet, 9 protons, m- and p- protons of the benzoyl groups) and 7.86 to 8.20 p.p.m. (multiplet, 6 protons, o- protons of the benzoyl groups).

Mass spectrum.

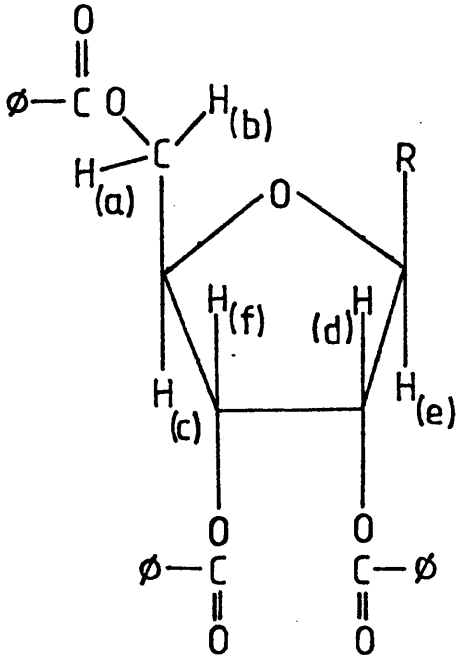
The interpreted mass spectrum is shown in figure 57.

1-Chloro-2,3,5-tribenzoyl-D-ribofuranose.

The above compound was prepared, according to the method of Davoll

Fig. 56.

Alphabetic assignments given to the protons on the ribofuranose ring and used in the interpretation of the n.m.r. of the compounds listed below.



Ø = benzoyl group.

(a) (R = CH_3CO):- 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose.

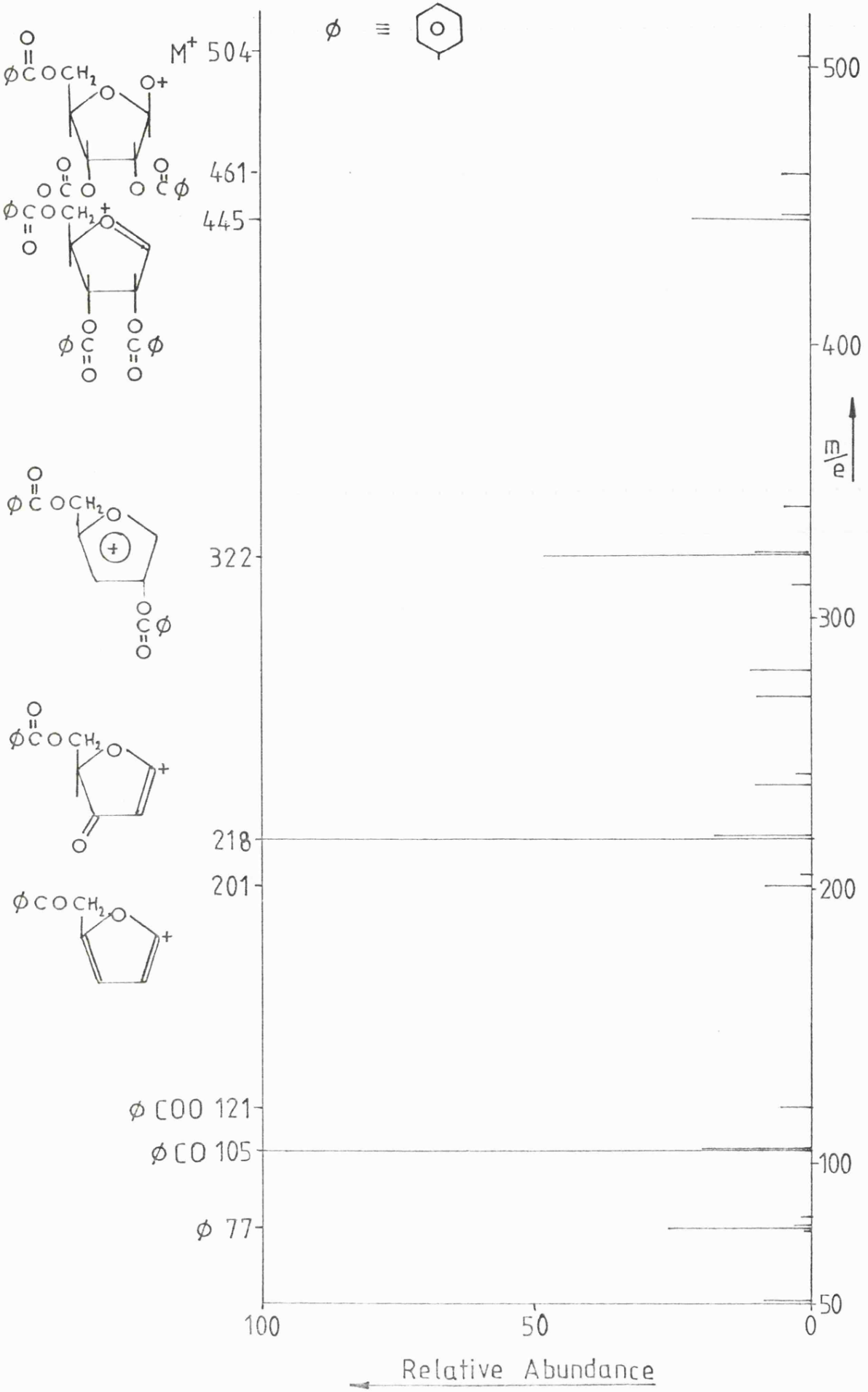
(b) (R = Cl):- 1-chloro-2,3,5-tri-O-benzoyl-D-ribofuranose.

(c) (R = nicotinamide):- N-(2',3',5'-tri-O-benzoyl-D-ribofuranosyl)-3-carbamoylpyridinium cation.

(d) (R = 3-trifluoroacetylpyridine):- N-(2',3',5'-tri-O-benzoyl-D-ribofuranosyl)-3-trifluoroacetylpyridinium cation.

Fig. 57.

Mass spectrum of 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose.



et. al. (1948), by treating 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose with HCl in dry diethyl ether. The product was isolated as a straw-coloured syrup (99% yield) and had the following spectroscopic data, which showed the product to be the desired compound, 1-chloro-2,3,5-tri-O-benzoyl-D-ribofuranose.

I.R. spectrum (liquid film).

Major absorptions:- 1735 (ester $\rangle C{=}{=}O$ of the benzoyl groups), 1475, 1450, 1315, 1265 ($\rangle C{-}{-}O$ stretch of the benzoyl groups), 1110, 1090, 702 and 678 cm^{-1} ($\rangle C{-}Cl$).

N.m.r. spectrum (in D^6-acetone).

The protons on the ribofuranose ring are given alphabetic assignments in figure 56. These assignments are used in the following table.

4.68 to 5.12 p.p.m. (multiplet, 3 protons, ribofuranosyl protons 'a', 'b' and 'c'), 5.76 to 6.13 p.p.m. (multiplet, 1 proton, ribofuranosyl proton 'f'), 6.55 p.p.m. (singlet, 1 proton, ribofuranosyl proton 'd'),6.87 p.p.m. (doublet, 1 proton, ribofuranosyl proton 'e'), 7.28 to 7.62 p.p.m. (multiplet, 9 protons, m- and p- protons of the benzoyl groups) and 7.92 to 8.22 p.p.m. (multiplet, 6 protons, o-protons of the benzoyl groups).

No resonances were observed at a chemical shift of 2.02 p.p.m. (the chemical shift of the acetyl group in the starting material).

Mass spectrum.

The interpreted mass spectrum is shown in figure 58.

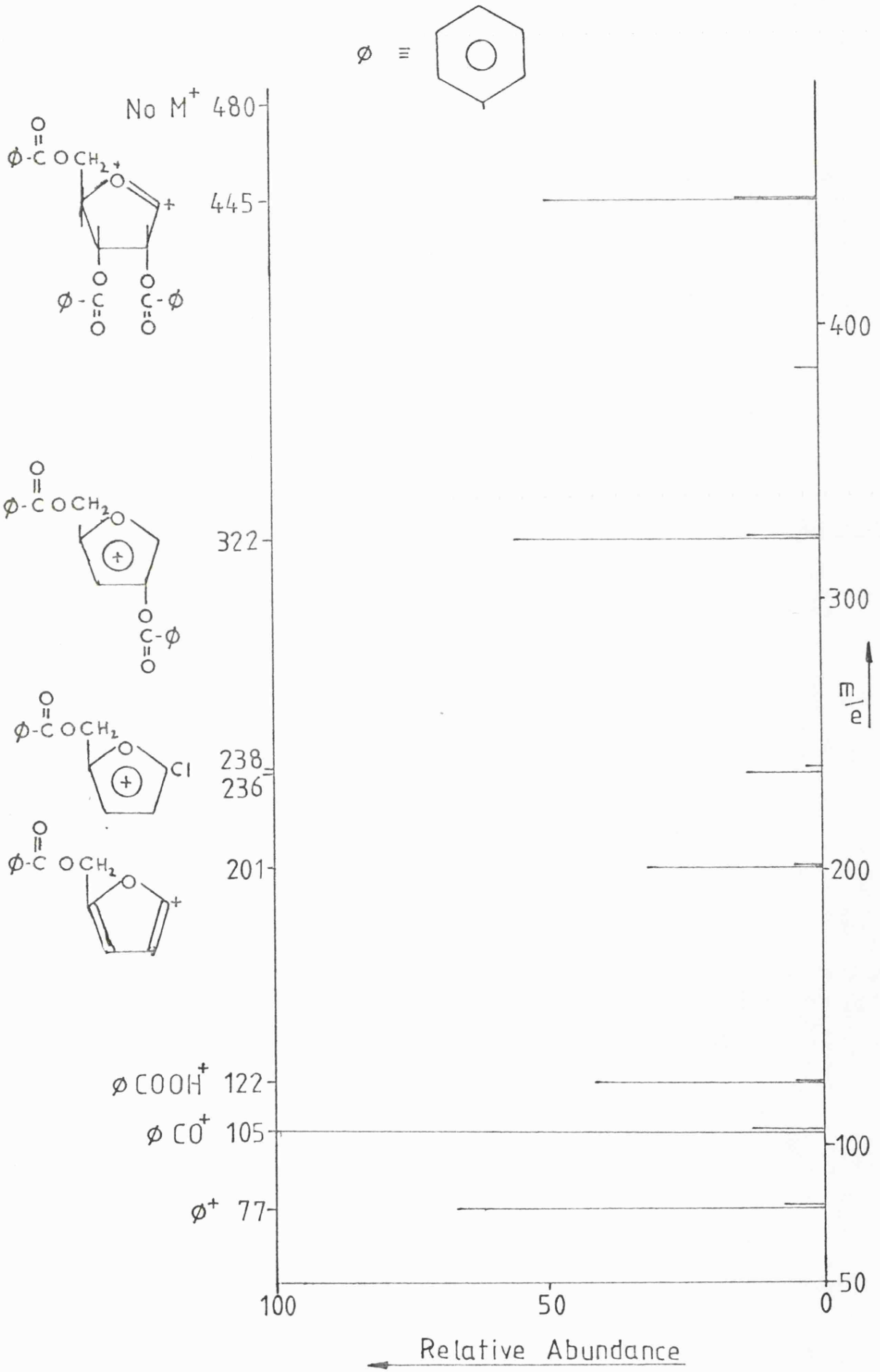
CHEMICAL SYNTHESIS OF NUCLEOSIDES AND NUCLEOTIDES.

N-(2',3',5'-Tri-O-benzoyl-D-ribofuranosyl)-3-trifluoroacetylpyridinium chloride.

The above compound was prepared by a method similar to that used by Haynes et. al. (1957) in their synthesis of NMN.

Fig. 58.

Mass spectrum of 1-chloro-2,3,5-tri-O-benzoyl-D-ribofuranose.



1-Chloro-2,3,5-tri-O-benzoyl-D-ribofuranose (0.025 moles) in acetonitrile (30 ml) and 3-trifluoroacetylpyridine (0.03 moles) in acetonitrile (100 ml) were mixed at 0°C and allowed to stand at this temperature for 48 hours. The precipitate was removed by filtration and the filtrate was evaporated to dryness in vacuo (at < 15°C /400 mm Hg) to yield a foam which was washed with dry diethyl ether. The residue was purified by dissolution in chloroform (15 ml) followed by reprecipitation with diethyl ether (200 ml) to give product A.

The precipitate, which had been removed from the reaction mixture by filtration, was dissolved in water and neutralised by the addition of saturated aqueous Na_2CO_3. The product was isolated by extraction with diethyl ether, dried ($MgSO_4$) and concentrated to dryness to give product B.

Product A was obtained as a buff-coloured powder (42% yield) the properties of which were consistent with its identification as the desired product, N-(2',3',5'-tri-O-benzoyl-D-ribofuranosyl)-3-trifluoroacetylpyridinium chloride.

I.R. spectrum (Nujol mull).

Major absorptions:- 1730 ($\rangle C{=}{=}O$ of the benzoyl groups and the ketonic $\rangle C{=}{=}O$), 1600, 1450, 1365, 1260 ($\rangle C{-}{-}O$ of the benzoyl groups), 1210 ($\rangle C{-}F$), 1180 ($\rangle C{-}F$), 1120 ($\rangle C{-}F$) and 710 cm^{-1}.

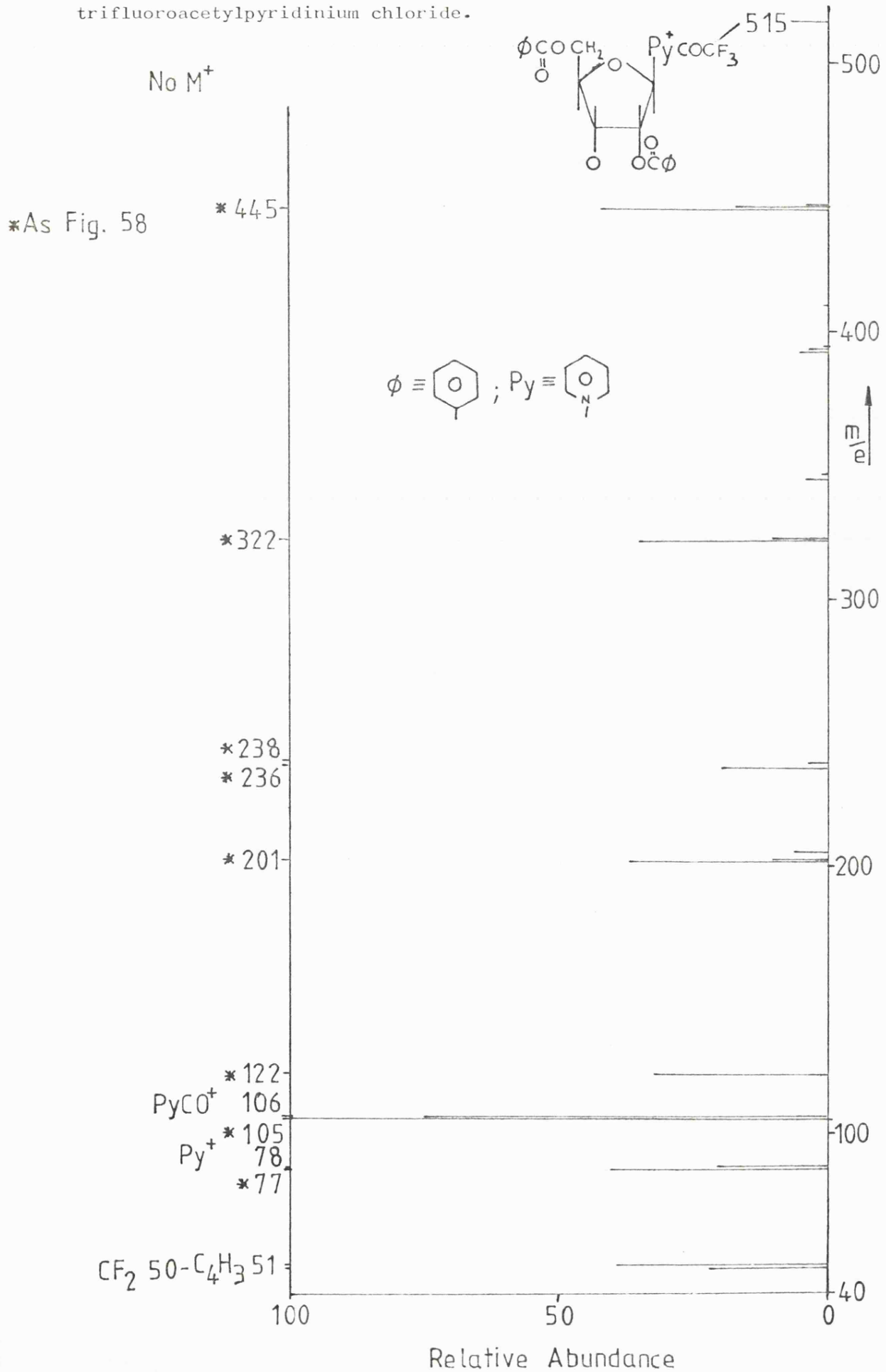
N.m.r. spectrum (in $CDCl_3$).

The protons on the ribofuranose ring are given alphabetic assignments in figure 56. These assignments are used in the following table.

^1H:- 4.30 to 5.14 p.p.m. (multiplet, 3 protons, ribofuranosyl protons 'a', 'b' and 'c'), 5.32 to 5.76 p.p.m. (multiplet, 2 protons, ribofuranosyl protons 'd' and 'f'), 6.06 p.p.m. (singlet, 1 proton, ribofuranosyl proton 'e'), 7.10 to 7.62 p.p.m. (multiplet, 9 protons, m- and p- protons of the benzoyl groups), 7.74 to 8.16 p.p.m.

Fig. 59.

Mass spectrum of N-(2',3',5'-tri-O-benzoyl-D-ribofuranosyl)-3-trifluoroacetylpyridinium chloride.



3.52 p.p.m.* (singlet, 1 proton), 5.02 p.p.m.* (singlet, 4 protons) 6.17 p.p.m.* (singlet, 1 proton, ribofuranosyl proton 'e'), 7.02 to 7.62 p.p.m. (multiplet, 9 protons, m- and p- protons on the benzoyl groups), 7.78 to 8.36 p.p.m. (multiplet, 8 protons+, o- protons on the benzoyl groups and the protons on the amidic $-NH_2$ group) and 8.48 to 9.63 p.p.m. (multiplet, 4 protons, protons on the pyridine ring).

*The spectrum was very poorly resolved, the resonances being very broad, and hence a detailed interpretation could not be made. However, the combined number of protons attributed to these resonances was equal to the number of protons on the ribofuranosyl ring.

+ On the addition of D_2O the integral of this resonance decreased to the equivalent of 6 protons, the protons of the amidic $-NH_2$ grouping having been deuteriated.

Mass spectrum.

The interpreted mass spectrum is shown in figure 60.

Product B (isolated as for product B above) was shown, by I.R. spectroscopy, to be nicotinamide.

N-(D-Ribofuranosyl)-3-carbamoylpyridinium chloride.

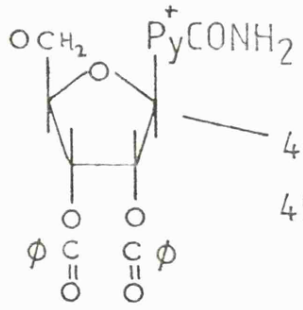
The above compound was prepared from the benzoylated nucleoside by treatment with methanolic ammonia according to the method of Haynes et. al. (1957). The required product, N-(D-ribofuranosyl)-3-carbamoyl-pyridinium chloride, was obtained as a pale yellow, hygroscopic powder (70% yield), and had the following spectroscopic data. Haynes et. al. quote a yield of 73%.

I.R. spectrum (Nujol mull).

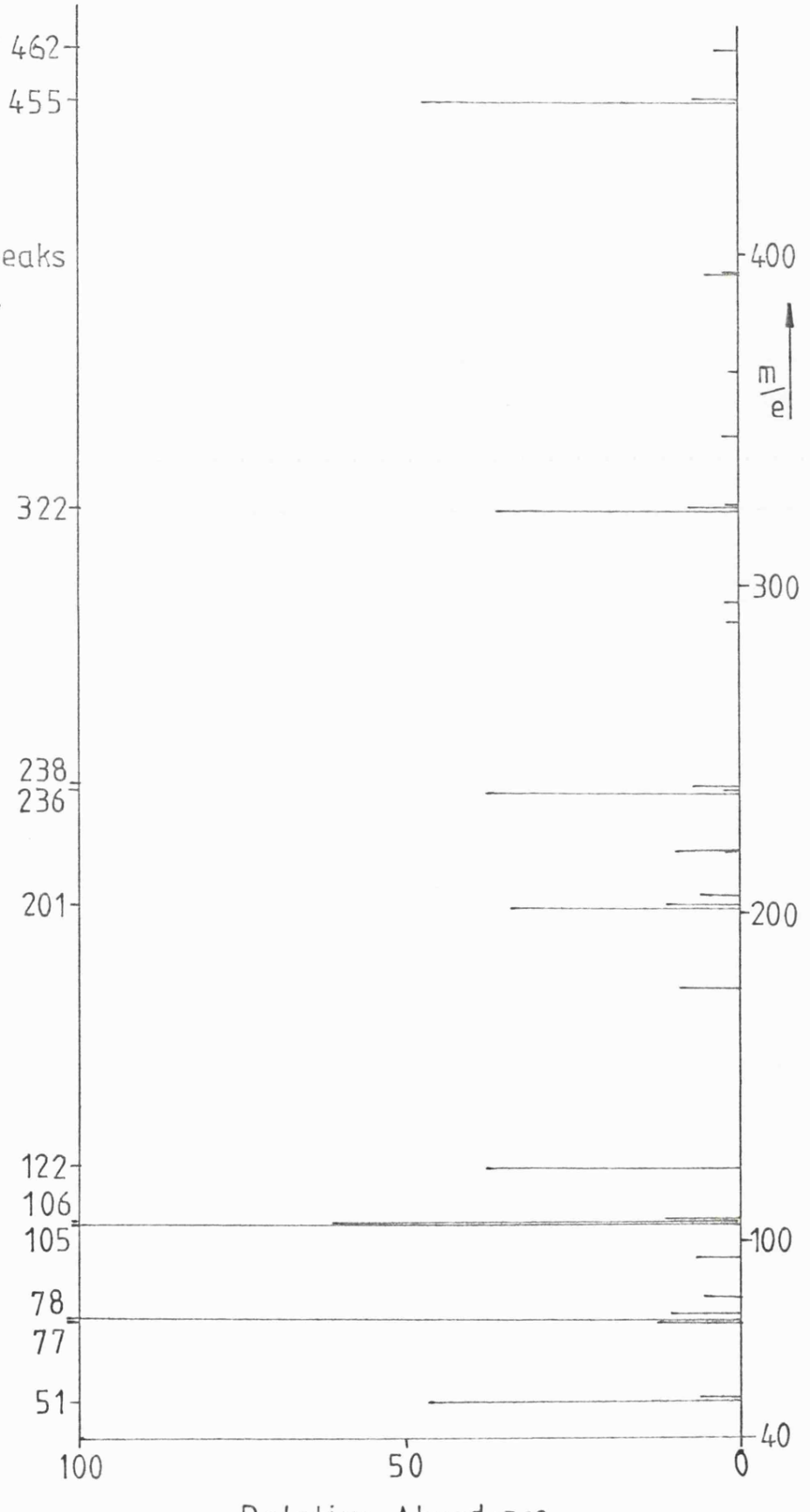
Major absorptions:- 3100 (broad, hydrogen bonded -OH), 1680 (amidic $\rangle C{=}{=}O$), 1590, 1540, 1230 and 960 cm^{-1}.

Fig. 60.

Mass spectrum of N-(2',3',5'-tri-O-benzoyl-D-ribofuranosyl)-3-carbamoylpyridinium chloride.



Remainder of peaks as for fig. 59.



N.m.r. spectrum (in D^6-DMSO).

The n.m.r. spectrum was a series of unresolved, broad resonances, and hence a detailed interpretation could not be undertaken. However, the following general observations may be made:-

3.20 to 6.60 p.p.m. (multiplet, 9 protons, $\geq$C—H and $\geq$C—OH protons on the ribofuranosyl moiety) and 7.80 to 9.70 p.p.m. (multiplet, 6 protons, protons on the pyridinium ring and the protons of the amidic $-NH_2$ group).

On the addition of D_2O the integrals of the ribofuranosyl protons: pyridinium protons:HDO changed to 6:4:5. This is consistent with the protons of the -OH groups and the amidic $-NH_2$ group having been deuteriated, as would be expected.

A test for carbohydrate proved positive.

N-(D-Ribofuranosyl)-3-trifluoroacetylpyridinium chloride.

Three methods were investigated in an attempt to debenzoylate N-(2',3',5'-tri-O-benzoyl-D-ribofuranosyl)-3-trifluoroacetylpyridinium chloride in order to obtain the above product.

(a) A similar method to that used to debenzoylate N-(2',3',5'-tri-O-benzoyl-D-ribofuranosyl)-3-carbamoylpyridinium chloride was used. N-(2',3',5'-Tri-O-benzoyl-D-ribofuranosyl)-3-trifluoroacetylpyridinium chloride (0.004 moles) and dry ammonia saturated methanol were allowed to stand for 18 hours. The solvents were removed in vacuo (at $< 10^{\circ}C$ / 400 mm Hg) to yield a yellow oil, which was dissolved in dry methanol (5 ml). The product was precipitated by the addition of ethyl acetate (220 ml), isolated by filtration (under a stream of nitrogen) and dried under vacuum to give a pale yellow hygroscopic powder (8% yield).

I.R. spectrum (Nujol mull).

Major absorptions:- 3100 (broad, hydrogen bonded -OH), 1720 (ketonic $\geq$C=O), 1595, 1530, 1490, 1220, 1190 ($\geq$C—F), 1165 ($\geq$C—F),

1120 ($\geq$C—F) and 730 cm^{-1}.

N.m.r. spectrum (in D^6-DMSO).

^1H:- The n.m.r. spectrum was a series of unresolved resonances, and hence a detailed interpretation could not be undertaken. However the following general observations may be made:-

3.71 to 6.20 p.p.m. (multiplet, 9 protons, $\geq$C—H and $\geq$C—OH protons on the ribofuranosyl moiety) and 7.90 to 9.50 p.p.m. (multiplet, 4 protons, protons on the pyridinium ring).

On the addition of D_2O the integrals of the ribofuranosyl protons: pyridinium protons:HDO changed to 6:4:3. This is consistent with the protons on the ribofuranosyl -OH groups having been deuteriated.

^{19}F:- 16.1 p.p.m. downfield (singlet).

Tests.

The product was tested for fluorine, carbohydrate and chloride, all of which proved positive.

(b) Sodium ethoxide (0.03 moles) in dry ethanol (200 ml) was reacted with N-(2',3',5'-tri-O-benzoyl-D-ribofuranosyl)-3-trifluoroacetyl-pyridinium chloride (0.01 moles) at 0°C for 48 hours. Dry 5% methanolic HCl (10 ml) was added to the reaction mixture and the solvents and excess HCl removed in vacuo (at < 10°C/400 mm Hg). The resultant gum was extracted with dry methanol (2 x 5 ml) and added to ethyl acetate (200 ml). Filtration (under an atmosphere of dry nitrogen) and drying yielded an off-white hygroscopic powder (25% yield), the infra-red spectrum of which was different from that of the product from preparation (a) in that it showed no $\geq$C—F absorptions. Chemical analysis for fluorine proved negative.

(c) N-(2',3',5'-Tri-O-benzoyl-D-ribofuranosyl)-3- trifluoroacetyl -pyridinium chloride (0.005 moles) was stirred with dry saturated methanolic HCl at 0°C for 24 hours. The solvents and excess HCl were

removed in vacuo (at $< 10^{o}C$ /400 mm Hg), the resultant gum was extracted with dry methanol (2 x 5 ml), the solution filtered and the product precipitated by the addition of ethyl acetate (200 ml). The precipitate was isolated by filtration and dried in vacuo to give product A. The mother liquor from the precipitation was evaporated to dryness to give product B.

Product A, obtained in 10% yield, was a yellow amorphous solid, the infra-red spectrum of which was similar to that of the product from method (b) above. The ^{19}F n.m.r. spectrum had no resonance due to fluorine, and a chemical test for fluorine also proved negative.

Product B, obtained in 15% yield,as a brown paste was shown, by infra-red spectroscopy, to contain 3-trifluoroacetylpyridine and its gem-diol, although these were not the only compounds present.

Phosphorylation of N-(D-ribofuranosyl)-3-carbamoylpyridinium chloride using o-phenylene phosphorochloridate.

A solution of o-phenylene phosphorochloridate (1.1 m-moles) in acetonitrile (2 ml) was added to a stirred suspension of N-(D-ribofuranosyl)-3-carbamoylpyridinium chloride (1 m-mole) and 2,6-lutidine (3m-moles) in acetonitrile (2 ml) at room temperature ($20^{o}C$) and the mixture was stirred for 3 hours, after which time water (1 ml) was added and the solvents removed in vacuo (at $< 25^{o}C$). The residue was dissolved in 0.2M-triethylammonium bicarbonate, pH 7.5 (50 ml) and 2% aqueous bromine was added. The solution was shaken for 20 minutes and then extracted with diethyl ether (10 ml portions) until the extracts were colourless. Air was bubbled through the aqueous layer, which was then concentrated to low bulk in vacuo (at $< 25^{o}C$). An aliquot (50 µl) of the reaction mixture was chromatographed on paper (Whatman No. 1) using an eluent of 1M-ammonium acetate,pH 5: ethanol (3:7). Several compounds were separated, which absorbed

ultra-violet light, but one (R_f = 0.29) had the same R_f as did NMN.

ENZYME ISOLATION AND ASSAY.

NAD^+ transglycosidase from pig brain.

This enzyme was isolated from whole pig brain using the method of Kaplan et. al. (1954).

Assay.

The enzyme solution (25 µl), 6 mM-NAD^+ solution (0.1 ml) and 0.1 M-KH_2PO_4 buffer, pH 7.5 (0.3 ml) were incubated at 37°C for 7.5 minutes. The decrease in NAD^+ concentration was measured by preparing the cyanide adduct (Colowick et. al.; 1951) and by assay using yeast alcohol dehydrogenase (Kaplan and Ciotti; 1954). By both methods the enzyme was found to contain 16 units ml^{-1} (1 unit ≡ 1 µ-mole of NAD^+ destroyed in one hour).

Nicotinamide phosphoribosyl transferase from Lactobacillus fructosus.

This enzyme was isolated by a modification of the method of Ohtsu and Nishizuka (1967).

Cells of Lactobacillus fructosus were grown in a medium of glucose (2 %), polypeptone (0.5 %), yeast extract (0.5 %), nicotinamide (10 mg l^{-1}) and fructose (1 %) in 0.01 M-potassium phosphate buffer, pH 7, at 26°C, harvested by centrifugation and washed with 0.14 M-KCl (2 x 50 ml). The cells were homogenised with cold acetone (40 ml) at -15°C and filtered rapidly on a Buchner funnel. The cell cake was brought to room temperature, crushed and dried under vacuum for two hours. The powder thus obtained was sufficiently stable to be stored at -15°C for an extended period.

The enzyme was purified for use by suspending the acetone powder (1 g) in 0.05 M-potassium phosphate buffer, pH 7 (20 ml) by stirring

for 15 minutes. The cells were disrupted in a sonic occillator for 20 minutes, and the resulting suspension was centrifuged at 12,000 x g and 4°C for 20 minutes. An equal volume of 0.4 %-protamine sulphate, pH 7, was added, the mixture was stirred for 30 minutes and the precipitate was removed by centrifugation (12,000 x g; 4°C). Ammonium sulphate (12 g) was slowly added to the supernatent and the precipitate was again removed by centrifugation (12,000 x g; 4°C). The pellet was dissolved in 0.05 M-potassium phosphate buffer, pH 7 (10 ml) and dialysed against 0.05 M-potassium phosphate, pH 7: 20 % aqueous polyethylene glycol (1:1) for 20 hours. The resultant solution was stored at -18°C until use, which was within 2 weeks.

Assay.

0.25 M-Tris acetate buffer, pH 7 (0.2 ml), 50 mM-PRPP (0.05 ml), 50 mM-ATP (0.05 ml), 50 mM-$MgCl_2$ (0.05 ml), 50 mM-nicotinamide (0.05 ml), 50 mM-7-^{14}C nicotinamide (5 µl, 50 mCi m-mole^{-1}), water (0.05 ml) and the enzyme preparation (0.05 ml) were incubated together at 37°C for 30 minutes. After this period the enzyme was denatured by the addition of ethanol (0.5 ml), and the protein was removed by centrifugation. An aliquot (200 µl)of the incubation mixture was analysed by t.l.c. on CM cellulose. Aliquots of NMN and nicotinamide solutions were also 'spotted' on to the plate to act as markers and the plate was developed by butan-1-ol:methanol:water:0.880 ammonia (60:20:20:1). The plate was dried, the nicotinamide 'spot' was visualised under ultra-violet light (R_f = 0.9) and the plate was redeveloped to R_f 0.85 with 1 M-acetic acid. The plate was dried, examined under ultra-violet light and the position of the NMN 'spot' (R_f = 0.8) was noted. The silica at the positions of nicotinamide and NMN on the sample chromatograph was scraped into scintallation

vials and 1.6 M-LiCl (0.4 ml) and scintallation fluid (2.5 ml) were added. The radioactivity due to each 'spot' was measured, and from this the activity of the enzyme was calculated, having determined the protein concentration by the method of Lowry et. al. (1951). The specific activity of the enzyme was found to be 0.0092 units mg^{-1}. Ohtsu and Nishizuka quote a specific activity of 0.0122 units mg^{-1}.

ENZYMIC SYNTHESIS.

3-Acetylpyridine adenine dinucleotide.

This compound was prepared by the method of Kaplan and Ciotti (1956), which used an exchange reaction between NAD^+ and 3-acetylpyridine, catalysed by NAD^+ transglycosidase from pig brain. The extent of the reaction was monitored by the reduction of the analogue and the residual NAD^+ by alcohol dehydrogenase from yeast. The reduced analogue had a λ_{max} at 365 nm compared to 340 nm for NADH itself. The maximal ratio of absorbance at 365 nm:340 nm (reduced analogue: reduced NAD^+) was reached after 3.5 hours and was 1.42:1. 3-Acetylpyridine adenine dinucleotide was isolated as a pale yellow amorphous powder (45 % yield). Kaplan and Ciotti quote a 40 % yield and a λ_{max} of 365 nm for the reduced analogue.

Attempted preparation of 3-trifluoroacetylpyridine adenine dinucleotide.

It was attempted to prepare this compound in a manner similar to that used to prepare 3-acetylpyridine adenine dinucleotide. NAD^+ transglycosidase from pig brain (ca. 2500 units), NAD^+ (2 g, Sigma Chemical Co.), 3-trifluoroacetylpyridine (1.5 g) and 1 M-potassium phosphate buffer, pH 7.5 (6.25 ml) were incubated at 20°C for 4 hours. The reaction was monitored by reaction with alcohol dehydrogenase from yeast or by the formation of the cyanide adduct. The peaks attributable to NADH or the NAD^+-cyanide adduct

decreased in size as the reaction proceeded, but no new peaks were observed. The protein was denatured by the addition of trichloroacetic acid (to 5 %) and was then removed by centrifugation. Acetone (5 volumes) was added to the supernatent and the precipitate was removed by centrifugation, dissolved in 0.1 M-sodium acetate (25 ml) and reprecipitated by the addition of acetone (125 ml). The precipitate was removed by centrifugation, washed with acetone (50 ml) and dried in vacuo to give a pale yellow amorphous powder (700 mg) which showed no ^{19}Fn.m.r.signal, nor was fluorine detected by a chemical test.

Chromatography on Whatman No. 4 paper using an eluent of ethanol: 0.1 M-acetic acid (1:1) showed that the product had an R_f of 0.55, which is the same as ADPR under these conditions.

In further attempts to prepare the analogue by the above method the pH of the buffer used was varied between 2 and 9 pH units, by preparing 2M-phosphoric acid and adding sufficient KOH to adjust the pH, and diluting the buffer to give a final concentration of 1 M. In no case was a fluorine-containing nucleotide isolated.

Inhibitory effect of 3-trifluoroacetylpyridine on NAD^+ transglycosidase from pig brain.

When an equal amount of 3-trifluoroacetylpyridine and 3-acetylpyridine were incubated with NAD^+ transglycosidase from pig brain in the same manner as above 3-acetylpyridine adenine dinucleotide was only formed at 35 % of the rate at which it was formed in the control experiment (with 3-acetylpyridine only).

3-Diazoacetylpyridine adenine dinucleotide.

This compound was prepared by an exchange reaction involving NAD^+, NAD^+ transglycosidase and 3-diazoacetylpyridine by the method of

Biellmann et. al. (1974), except that the excess NAD^+ was not destroyed by the addition of NAD^+ glycohydrolase from Neurospora Crassa.

The product was isolated as an off-white powder (23 % yield). The cyanide adduct had a λ_{max} of 365 nm. Biellmann quotes a yield of 13 % and a λ_{max} for the cyanide adduct of 365 nm.

Assay with alcohol dehydrogenase from yeast showed that the product contained some 18 % residual NAD^+ (the analogue is not reduced by alcohol dehydrogenase from yeast).

3-Chloroacetylpyridine adenine dinucleotide.

This compound was prepared by the action of chloride ions on 3-diazoacetylpyridine adenine dinucleotide according to the method of Biellmann et. al. (1974). The product was obtained as an amorphous powder (73 % yield) which, when reduced with alcohol dehydrogenase from horse liver, had a λ_{max} of 375 nm. Biellmann quotes a λ_{max} for the reduced analogue of 374.5 nm.

Attempted preparation of 3-fluoroacetylpyridine adenine dinucleotide.

3-Diazoacetylpyridine adenine dinucleotide (250 μ-moles), lithium fluoride (20 m-moles) and hydrogen fluoride (2 m-moles) were stirred in water (10 ml) for 4 hours. The excess lithium and fluoride ions were removed by cation and anion exchange chromatography respectively. The eluent was liophilised to give an amorphous powder (25 mg) which gave no $^{19}F_{n.m.r.}$ signal. Fluorine could not be detected chemically.

Investigation of the effect of 3-trifluoroacetylpyridine on nicotinamide phosphoribosyl transferase from Lactobacillus fructosus.

0.25 M-Tris acetate buffer, pH 7 (0.2 ml), 50 mM-PRPP (0.05 ml), 50 mM-ATP (0.05 ml), 0.25 M-$MgCl_2$ (0.05 ml), water (0.05 ml) and the enzyme preparation (0.05 ml) were incubated with:-

(a) 50 mM-nicotinamide (0.05 ml)

(b) 50 mM-3-trifluoroacetylpyridine (0.05 ml)

(c) 50 mM-nicotinamide (0.025 ml) + 50 mM-3-trifluoroacetylpyridine (0.025 ml)

(d) as in (a) but with 7-^{14}C-nicotinamide (2 µl)

(e) as in (b) but with 7-^{14}C-nicotinamide (2 µl)

at 37°C for 4 hours, after which time the enzyme was denatured by the addition of ethanol (0.5 ml) and removed by centrifugation. The samples were analysed by t.l.c. under the same conditions as were used in the assay of the enzyme, and the radioactivity due to the starting material and the product was counted for incubations (d) and (e). It was found that in all the incubations a compound with a similar R_f to NMN had been formed. Additionally the proportion of the radioactivity associated with the NMN 'spots' of incubations (d) and (e) was approximately the same, the ratio of nicotinamide:NMN being 0.32 in incubation (d) and 0.28 in incubation (e).
An aliquot of incubation mixture (b) was examined by ^{19}F n.m.r. spectroscopy and a new ^{19}F resonance was noted, the signal being a singlet and 8.70 p.p.m. upfield from trifluoroacetic acid. A signal attributable to 3-trifluoroacetylpyridine was also noted.

Enzymic pyrophosphorylation.

A mixture of 0.25 M-tris acetate buffer, pH 5.8(0.2 ml), 100 mM-ATP (0.05 ml), 2-^{3}H-ATP,ammonium salt(2 µl, supplied in 50 % aqueous ethanol, activity = 15 to 30 Ci m-mole^{-1}), 0.1 M-$MgCl_2$(0.05 ml), water(0.05 ml),NAD^+ pyrophosphorylase(0.05 ml, from hog liver), and inorganic pyrophosphatase(5 µl, from bakers yeast, supplied by the Sigma chemical company) were incubated with:-

(a) 50 mM-NMN(0.05 ml)

(b) 50 mM-3-trifluoroacetylpyridine mononucleotide (0.05 ml, crude, obtained as above)

at 37°C for 90 minutes, and after this time the protein was denatured by the addition of ethanol(0.5 ml) and removed by centrifugation. The supernatent was analysed by t.l.c. on PEI cellulose using 0.1 M-acetic acid as the eluent. The compounds which were eluted were visualised under ultra-violet light and those attributable to ATP and NAD^+(or its analogue) were scraped into scintallation vials, 1.6 M-lithium chloride(0.4 ml) and scintallation fluid(2.5 ml) were added and the radioactivity of each sample measured.

Incubation (a) showed zones corresponding to NAD^+(R_f = 0.41) and ATP(R_f = 0.04) with radioactivities of 1315 c.p.m. and 54,414 c.p.m. respectively. This is equivalent to 0.14 μ-moles out of a possible 2.5 μ-moles of NAD^+ having been formed.

Incubation (b) showed zones corresponding to 3-trifluoroacetylpyridine adenine dinucleotide(R_f = 0.39) and ATP(R_f = 0.04) with radioactivities of 10,048 c.p.m. and 65,209 c.p.m. respectively. This is equivalent to 1.04 μ-moles out of a possible 2.5 μ-moles of 3-trifluoroacetylpyridine adenine dinucleotide having been formed.

<u>One-stage enzymic synthesis of 3-trifluoroacetylpyridine adenine dinucleotide and NAD^+.</u>

A mixture of 0.25 M-tris acetate, pH 7(0.2 ml), 50 mM-PRPP(0.05 ml), 100 mM-ATP(0.05 ml), 0.25 M-$MgCl_2$(0.05 ml), 2-^{3}H-ATP, ammonium salt (5 μl), nicotinamide phosphoribosyl transferase (0.05 ml, from <u>Lactobacillus fructosus</u>), NAD^+ pyrophosphorylase (0.05 ml, from hog liver), inorganic pyrophosphatase (5 μl, from bakers yeast) and:-

(a) 50 mM-nicotinamide (0.05 ml)

(b) 3-trifluoroacetylpyridine (0.05 ml)

were incubated together at 37°C for 4 hours, after which time the enzymes were denatured by the addition of ethanol (0.5 ml) and removed by centrifugation.

An aliquot of the supernatent (50 µl) was analysed by t.l.c. on PEI cellulose plates. In order to facilitate a good separation of the nucleotides the plate was developed 2 cm past the origin with 0.2 M-LiCl, for a further 3 cm with 1.0 M-LiCl and then the plate was fully eluted with 1.6 M-LiCl. The plate was divided into strips at ½ cm intervals along the solvent path and each of these strips was placed into a scintallation vial along with 1.6 M-LiCl (0.4 ml) and scintallation fluid (2.5 ml) and the radioactivity of each was measured. From graphs of radioactivity (c.p.m.) against distance up the t.l.c. plate (cm) (figs. 61 and 62) it was calculated that 1.59 µ-moles of NAD^+ and 1.72 µ-moles of 3-trifluoroacetylpyridine adenine dinucleotide had been formed, out of a possible 2.5 µ-moles in each case.

Investigation into the base-specificity of nicotinamide phosphoribosyl transferase from Lactobacillus fructosus.

The specificity of this enzyme was investigated by incubating:-

(a) 50 mM-nicotinamide (0.05 ml)

(b) 50 mM-3-trifluoroacetylpyridine (0.05 ml)

(c) 50 mM-nicotinic acid (0.05 ml)

(d) 50 mM-3-acetylpyridine (0.05 ml)

(e) 50 mM-ethyl nicotinoylacetate (0.05 ml)

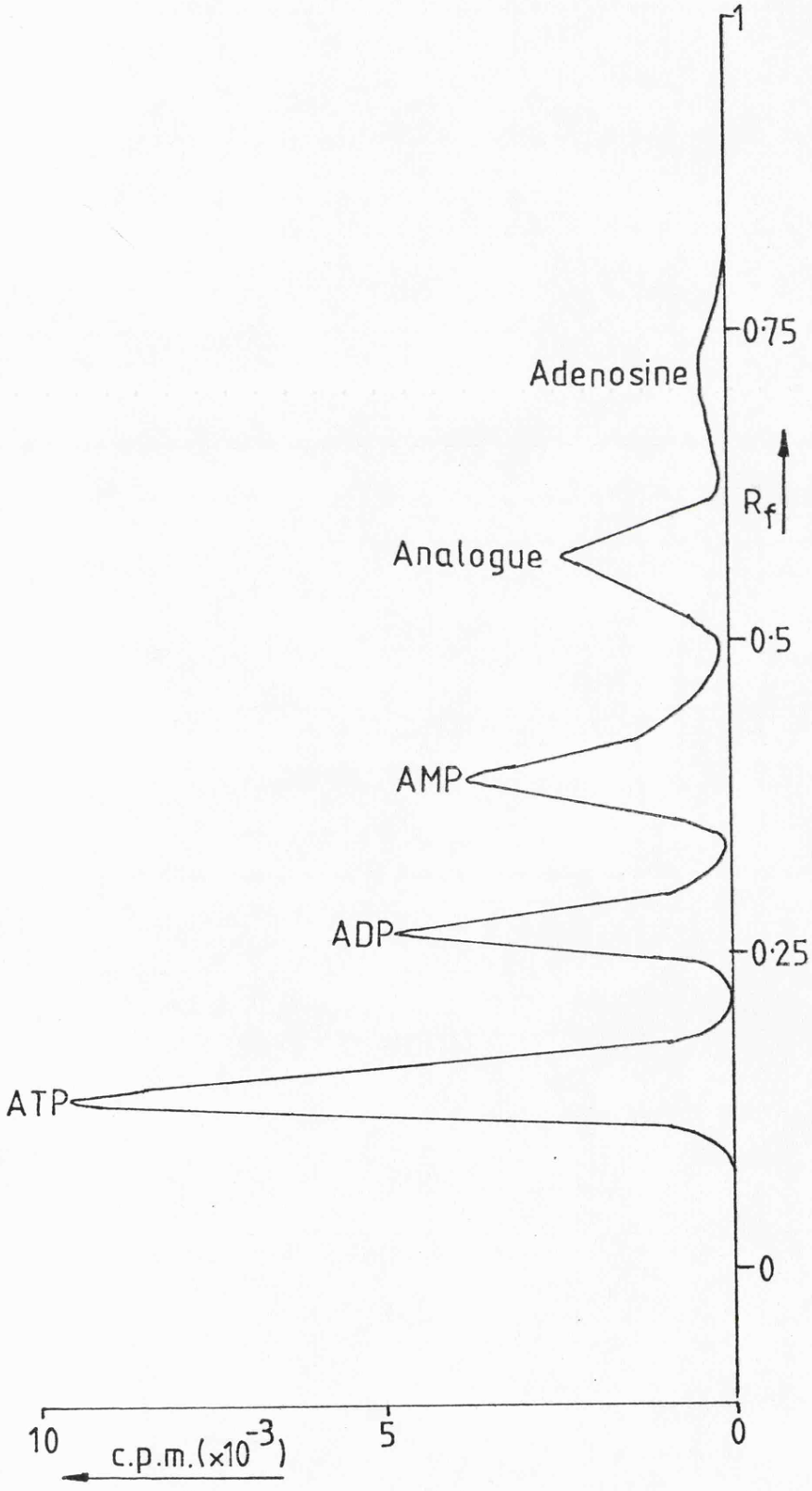
(f) 50 mM-pyridin-3-al (0.05 ml)

(g) 50 mM-3-bromopyridine (0.05 ml)

in a similar manner to that used in the initial investigation of this

Fig. 62.

Radioactivity profile of the one-stage enzymic synthesis of 3-trifluoroacetylpyridine adenine dinucleotide.



enzyme (see page 86), aliquots (50 µl) of the reaction mixture being analysed by t.l.c. as before. The remainder of the products were lyophilised and retained for use in the next experiment.
It was found that incubations (a), (b), (d) and (f) contained a compound with a similar R_f to NMN under the conditions of assay which were used.

Specificity of enzymic pyrophosphorylation.

The enzymic pyrophosphorylation of the products from the previous experiment was attempted in a similar manner to that used before (see page 87) and t.l.c. analysis of the products again undertaken. Incubations (a), (b), (d) and (f) were found to have incorporated 2-^{3}H-ATP into a product with a similar R_f to NAD^+.

Speed of formation of 3-trifluoroacetylpyridine adenine dinucleotide.

A mixture of 0.25 M-tris acetate, pH 6.5 (0.4 ml), 50 mM-PRPP (0.1 ml), 100 mM-ATP (0.2 ml), 0.25 M-$MgCl_2$ (0.1 ml), 50 mM-3-trifluoroacetylpyridine (0.1 ml), NAD^+ pyrophosphorylase (0.05 ml, from hog liver), inorganic pyrophosphatase (5 µl, from bakers yeast) and 2-^{3}H-ATP, ammonium salt (5 µl) were equilabrated to a temperature of 37°C. An aliquot (50 µl) of the mixture was taken (to act as T = 0) and ethanol (50 µl) was added, the mixture being stored under refridgeration until analysis. Nicotinamide phosphoribosyl transferase (0.05 ml, from Lactobacillus fructosus) was added to initiate the reaction and further aliquots (50 µl) taken at times of 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6 and 10 hours and treated in a similar manner to the original aliquot.
The aliquots were analysed by t.l.c. on PEI cellulose with an eluent of various concentrations of lithium chloride, as described previously (see page 89). The results obtained are expressed graphically in the form of concentration against time for ATP, ADP, AMP,

3-trifluoroacetylpyridine adenine dinucleotide and adenosine (Fig. 63).

FINAL PROCEDURES ADOPTED FOR THE PREPARATION AND PURIFICATION OF THE 3-TRIFLUOROACETYLPYRIDINE ANALOGUES OF NMN AND NAD^+.

3-Trifluoroacetylpyridine mononucleotide.

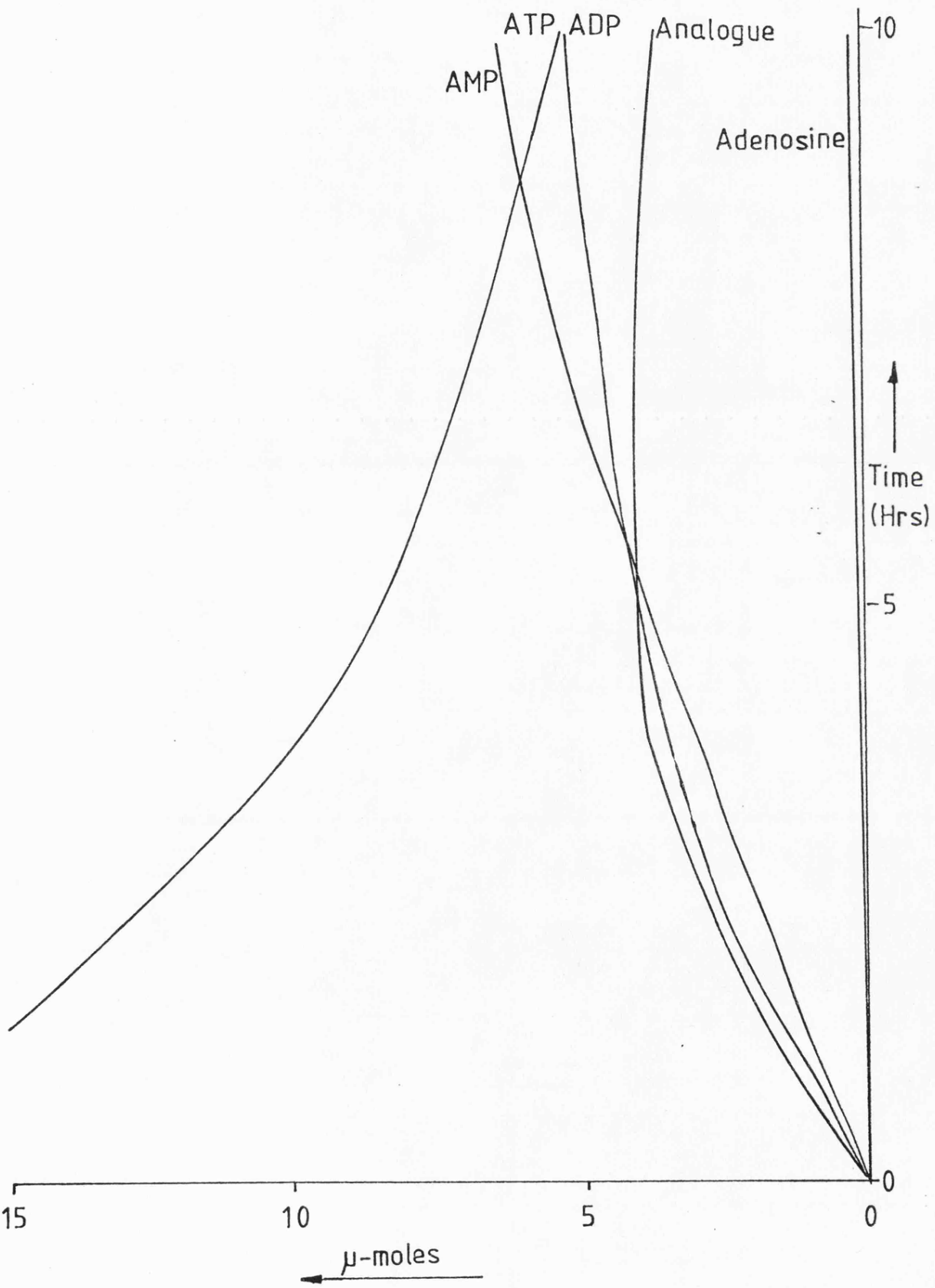
A mixture of 0.25 M-tris acetate, pH 7 (4 ml), 100 mM-ATP (1 ml), 50 mM-PRPP (1 ml), 50 mM-3-trifluoroacetylpyridine (1 ml), 0.25 M-$MgCl_2$ (1 ml), water (1 ml) and nicotinamide phosphoribosyl transferase (1 ml, from Lactobacillus fructosus) were incubated at 37°C for 2 hours, after which time the protein was denatured by the addition of ethanol (10 ml) and removed by centrifugation. The solution was lyophilised, the solids were washed with ethanol (15 ml), the insoluble material was dissolved in water (8 ml) and chromatographed on a Dowex 1X8-acetate form column (2 cm x 2.5 cm dia., BioRad Ltd.). The column was washed with water (50 ml) to remove the inorganic salts and the product, 3-trifluoroacetylpyridine mononucleotide was eluted from the column with 0.5 M-acetic acid (50ml) and lyophilised to give a white amorphous powder.

3-Trifluoroacetylpyridine adenine dinucleotide.

A mixture of 0.25 M-tris acetate, pH 6.5 (4 ml), 0.25 M-$MgCl_2$ (1 ml), 50 mM-trifluoroacetylpyridine (1 ml), 150 mM-ATP (1 ml), 50 mM-PRPP (1 ml), 2-^{3}H-ATP (25 µl), nicotinamide phosphoribosyl transferase (1 ml, from Lactobacillus fructosus),NAD^+ pyrophosphorylase (0.5 ml, from hog liver) and inorganic pyrophosphatase (50 µl, from bakers yeast) was incubated at 37°C for 4 hours, after which time the protein was denatured by the addition of ethanol (10 ml) and removed by centrifugation. The mixture was diluted with water (50 ml) and lyophilised. The residue was dissolved in 0.1 M-potassium phosphate

Fig. 63.

Speed of formation of 3-trifluoroacetylpyridine adenine dinucleotide in its one-stage enzymic synthesis.



buffer, pH 7 (10 ml) and incubated with alkaline phosphatase (5 µl, from calf intestine) at 37°C for 15 minutes. T.l.c. on PEI cellulose using an eluent of lithium chloride under the same conditions as before (see page 89) showed that all the AMP had been destroyed in this reaction (Fig. 64). The reaction mixture was applied to a Dowex 1X8-acetate form column (5 cm x 2.5 cm dia.) which was washed with water(150ml). The product, 3-trifluoroacetylpyridine adenine dinucleotide, was washed from the column with 1 M-acetic acid (150 ml) and the eluent was lyophilised to give a white amorphous powder. (It was shown in later experiments that 90% of the product was eluted with 100 ml of 1 M-acetic acid and 98% with 150 ml.)

It was shown, by the amount of incorporation of 2-^{3}H-ATP into the product, that the yield of 3-trifluoroacetylpyridine adenine dinucleotide was 75%, based on 3-trifluoroacetylpyridine. This yield was confirmed spectroscopically, based on a measurement of the relative absorption at 260 and 266 nm, assuming, as was later confirmed, the analogue to have a similar λ_{max} and extinction coefficient to NAD^+.

ANALYSIS OF 3-TRIFLUOROACETYLPYRIDINE MONONUCLEOTIDE AND 3-TRIFLUOROACETYLPYRIDINE ADENINE DINUCLEOTIDE.

The fluoro-analogues of NMN and NAD^+ thus prepared were analysed by the following methods, the results for assays (a) to (d) being given in Figure 65.

(a) 3-Trifluoroacetylpyridine content.

The glycosidic bond of the analogue under investigations (10 µ-moles) was cleaved by reaction with 2 N-NaOH (5 ml) and the 3-trifluoroacetylpyridine liberated was extracted with diethyl ether (5 x 5 ml). The extracts were dried ($MgSO_4$), evaporated to dryness in vacuo and the 2, 4-dinitrophenylhydrazone formed as before (see page 73) except

Fig. 64.

Radioactivity profile of the one-stage enzymic synthesis of 3-trifluoroacetylpyridine adenine dinucleotide after incubation with alkaline phosphatase.

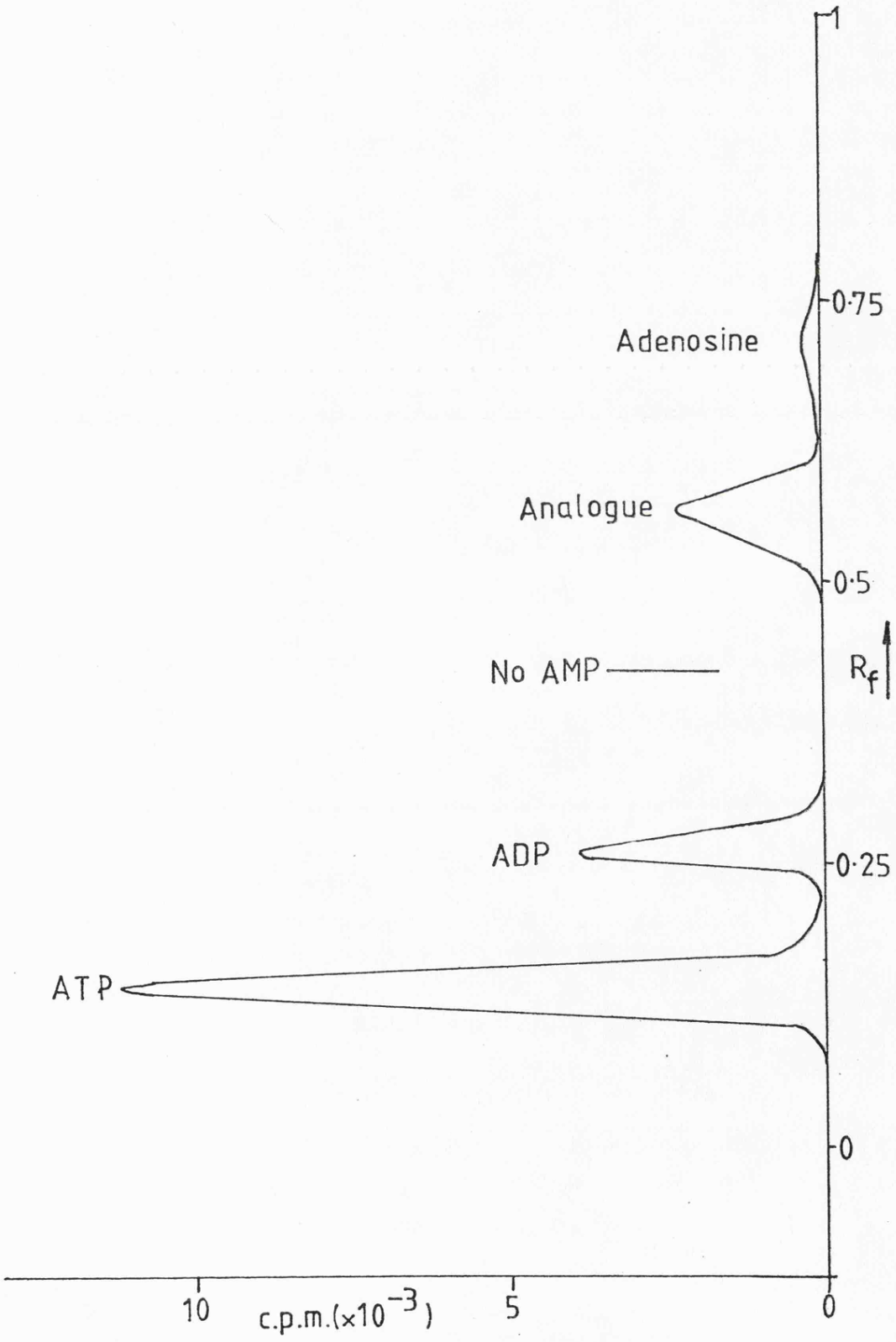


Fig. 65.

Analytical data on 3-trifluoroacetylpyridine mononucleutide and 3-trifluoroacetylpyridine adenine dinucleotide.

Component	NMN analogue	NAD^+ analogue
3-trifluoroacetylpyridine	0.89	0.94
ribose	1.05	1.95
phosphate	0.93	1.92
adenylic acid	-	1.03

Results are given in μ-moles of component per μ-mole of analogue.

that it was not recrystallised, but dissolved in chloroform (1 ml) and applied to a magnesia:celite (3:1) column (1 cm x 0.5 cm dia.). The hydrazone was eluted by chloroform (10 ml), the eluent evaporated to 1 ml and the optical density at 375 nm measured.

In later experiments the average yield of the hydrazone, prepared from 3-trifluoroacetylpyridine itself (10 μ-moles) and purified by the above procedure was 72 % and hence the results of the determination of the 3-trifluoroacetylpyridine content of the analogues were corrected using this figure.

(b) Ribose.

This was determined using the orcinol procedure (Taylor et. al.;1948).

(c) Total phosphate.

This was determined by the method of Bartlett (1959).

(d) Adenylic acid.

This was determined on 3-trifluoroacetylpyridine adenine dinucleotide only by reaction with 5'-adenylic acid deaminase (Colowick; 1955) after cleavage of the analogue with NAD^+ pyrophosphatase from snake venom (Shuster et. al.; 1955).

(e) Thin layer chromatography.

3-Trifluoroacetylpyridine mononucleotide was chromatographed on CM cellulose in a similar manner to that used in the assay of nicotinamide phosphoribosyl transferase (see page 83).

A single 'spot' (R_f = 0.80) was observed when the plate was examined under ultra-violet light. This is a similar R_f to NMN.

3-Trifluoroacetylpyridine adenine dinucleotide was chromatographed:-

(i) on PEI cellulose using an eluent of 1 M-acetic acid. Only one 'spot' was found, both by examining the radioactivity profile or by examining the plate under ultra-violet light. This 'spot' had an

R_f of 0.40, which is identical to that of NAD^+.

(ii) on PEI cellulose, using the eluent detailed previously (see page 89). Again only one 'spot' was detected, both by radioactivity and by ultra-violet light, and this had an R_f of 0.57, which is the same as NAD^+.

(f) ^{19}F Nuclear magnetic resonance spectrum.

3-Trifluoroacetylpyridine mononucleotide had a single resonance at 8.70 p.p.m. upfield from trifluoroacetic acid, whereas 3-trifluoroacetylpyridine adenine dinucleotide had a single resonance at 7.28 p.p.m. upfield from trifluoroacetic acid.

(g) Ultra-violet spectrum.

3-Trifluoroacetylpyridine adenine dinucleotide had a λ_{max} at 259 nm and an extinction coefficient of 17.5 cm^2 μ-$mole^{-1}$.

INVESTIGATION OF THE PROPERTIES OF THE ANALOGUES.

Stability of 3-trifluoroacetylpyridine adenine dinucleotide at various pH values.

The stability of this analogue was determined at pH 4, 5, 6, 7, 8 and 9 in aqueous solution, the pH of the solution being kept constant by the addition of 0.01 M-NaOH. The amount of analogue hydrolysed was determined by t.l.c. under the conditions previously detailed (see page 89). The radioactivity corresponding to the remaining analogue was measured, as was that due to the decomposition products. The experiments were all performed at 25°C.

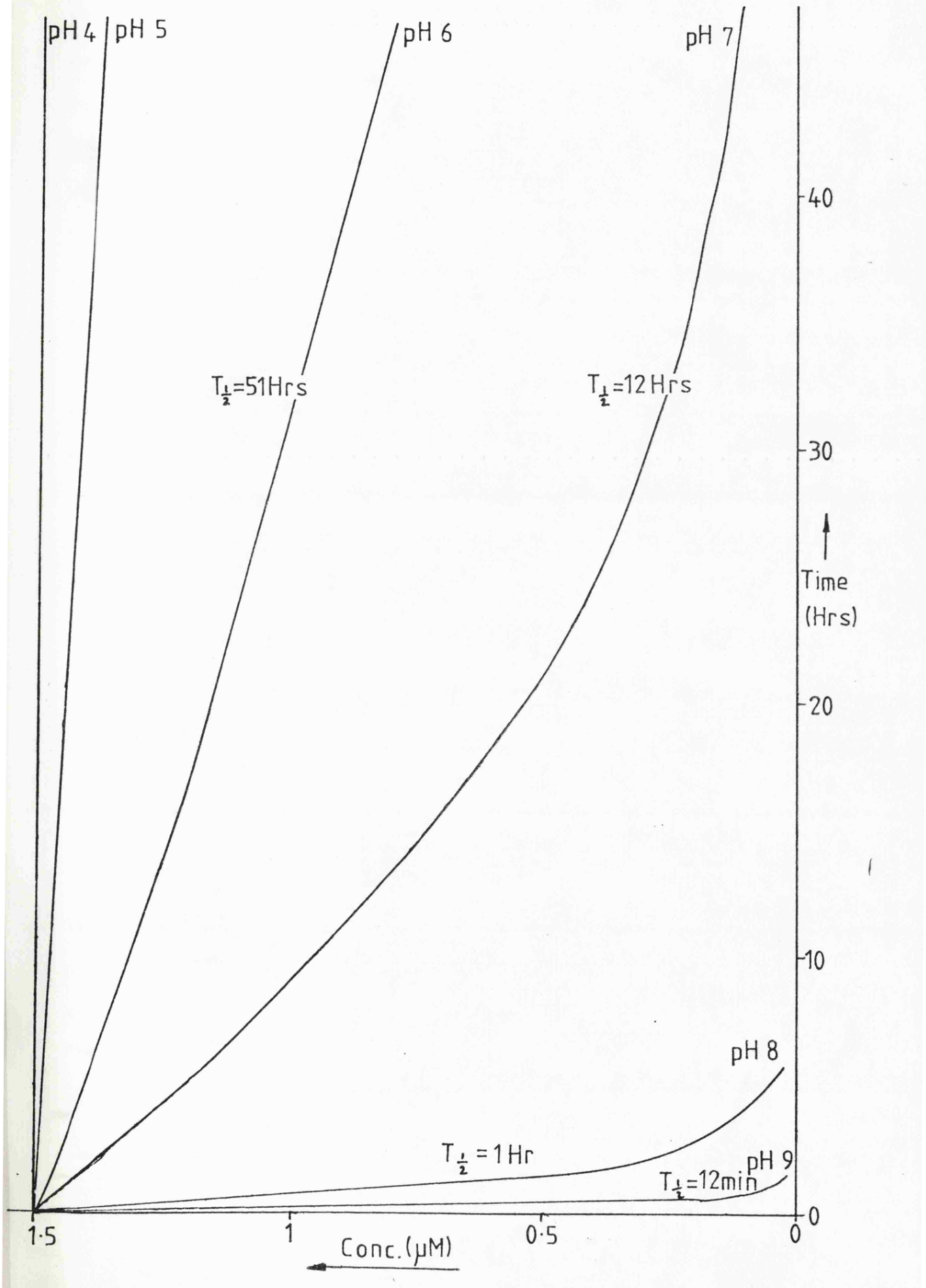
Plots of time against amount of analogue remaining are shown in figure 66, as are the half-lifes at the various pH values.

Reaction of the analogues with cyanide ions.

Any changes in the ultra-violet spectrum of a 20 mM solution of the analogue (0.2 ml) and 1 M-KCN, pH 11 (3 ml)were monitored over the

Fig. 66.

Rate of decomposition of 3-trifluoroacetylpyridine adenine dinucleotide at various pH values.



wavelength range 300 to 700 nm (against 1 M-KCN blank).

No changes in the spectrum of either 3-trifluoroacetylpyridine mononucleotide or 3-trifluoroacetylpyridine adenine dinucleotide were noted.

Chemical reduction of the analogues.

Water (1.8 ml), a 50 mM solution of the analogue (0.2 ml) and 0.2 M-sodium cyanoborohydride (100 µl, in 0.2 M-tris acetate, pH 7) were mixed, and any changes in the spectrum, over the wavelength range 300 to 500 nm noted.

No new absorption in the spectrum of either analogue was noted.

Co-enzymic activity of 3-trifluoroacetylpyridine adenine dinucleotide.

The ability of this analogue to mimic NAD^+ in several dehydrogenase systems was investigated.

Alcohol dehydrogenase from yeast.

A mixture of alcoholic tris buffer (a mixture of 0.1 M-tris(hydroxymethyl)aminomethane and 0.5 M-ethanol), pH 10.1 (2.5 ml), a 30 mM solution of the analogue (0.2 ml)was prepared and alcohol dehydrogenase from yeast (10 µl, 1:100 dilution) was added. The spectrum over the wavelength range 300 to 600 nm was measured before and after the addition of the enzyme.

No changes in the spectrum were noted.

Similarly, no co-enzymic activity was noted with alcohol dehydrogenase from horse liver, malate dehydrogenase from pig heart mitochondria, lactate dehydrogenase from rabbit muscle or glyceraldehyde-3-phosphate dehydrogenase from yeast.

Investigation of the inhibitory effect of 3-trifluoroacetylpyridine adenine dinucleotide.

(a) Alcohol dehydrogenase (from yeast).

Glycine-sodium pyrophosphate buffer, pH 9 (2.5 ml, 1.7 g l^{-1} glycine in 75 mM-sodium pyrophosphate), 2.2 M-semicarbazide, pH 6.5 (0.1 ml), ethanol (0.1 ml), 27.7 mM-NAD^+ (0.2 ml) and 0.3 M-glutathione (0.1 ml, reduced form) were incubated with alcohol dehydrogenase from yeast (10 µl, 1:5000 dilution) at 25°C, and the initial rate of the reaction was measured by monitoring the rate of increase in NADH concentration (measured at 340 nm).

The reaction was repeated, except that 3-trifluoroacetylpyridine (to a final concentration of 1 mM in the reaction medium) was added.

Result:-

Rate of reaction with the inhibitor added = 89 % of the rate without inhibitor (average of 3 determinations).

(b) Lactate dehydrogenase (from hog muscle).

0.1 M-Potassium phosphate buffer, pH 7.0 (2.83 ml), 23 mM-sodium pyruvate (0.1 ml) and 12 mM-NADH (0.05 ml) were incubated with lactate dehydrogenase (10 µl, 1:1000 dilution) at 25°C, and the initial rate of reaction was measured by monitoring the rate of decrease in NADH concentration (measured at 340 nm).

The reaction was repeated, except that 3-trifluoroacetylpyridine (to a final concentration of 1 mM in the reaction medium) was added.

Result:-

Rate of reaction with the inhibitor added = 78 % of the rate without inhibitor (average of 3 determinations).

(c) Glyceraldehyde-3-phosphate dehydrogenase (from yeast).

0.1 M-Potassium phosphate buffer, pH 7.1 (2.1 ml), 27.7 mM-NAD^+ (0.2ml) and glyceraldehyde-3-phosphate (0.2 ml) were incubated with glyceraldehyde-3-phosphate dehydrogenase (10 µl, 1:100 dilution) at 25°C, and the initial rate of the reaction was measured by monitoring the rate of increase in NADH concentration (measured at 340 nm).

The reaction was repeated, except that 3-trifluoroacetylpyridine (to a final concentration of 1 mM in the reaction medium) was added.

Result:-

Rate of reaction with the inhibitor added = 91 % of the rate without inhibitor (average of 3 determinations).

(d) Malate dehydrogenase (from pig heart mitochondria)

100 mM-Sodium malate (10 µl), 36 mM-NAD^+ (10 µl) and malate dehydrogenase (10 µl, 1:100 diution, a gift from Dr. G. Smith) in 50 mM-triethanolamine buffer, pH 7.8 to a total volume of 1 ml were incubated at 25°C , and the initial rate of the reaction was measured by monitoring the rate of increase in NADH concentration (measured at 340 nm).

The reaction was repeated, except that 3-trifluoroacetylpyridine (to a final concentration of 0.36 mM in the reaction medium) was added.

Result:-

Rate of reaction with the inhibitor added = 62 % of the rate without inhibitor (average of 3 determinations).

All reactions with dehydrogenases were carried out at a temperature of 25°C.

RESULTS

and

DISCUSSION

Results and Discussion.

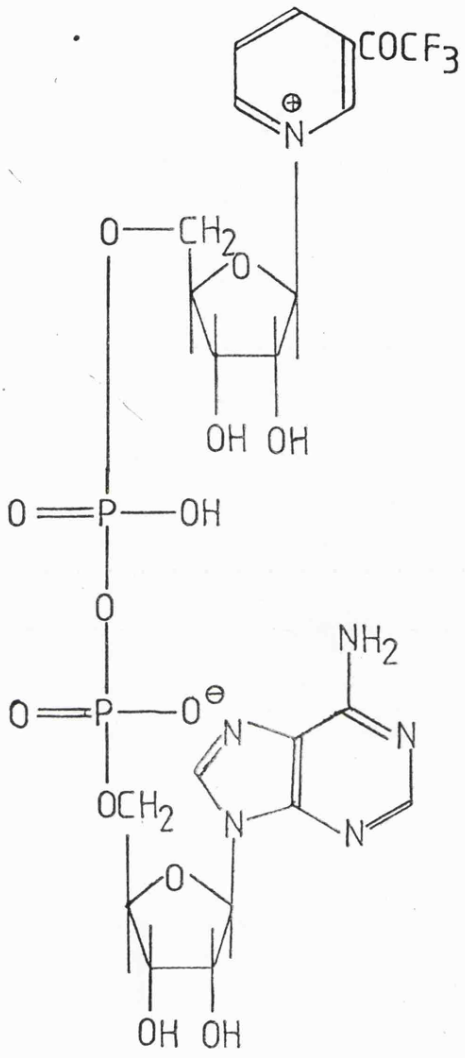
In view of the wide involvement of NAD^+ in enzymically catalysed reactions a fluorinated analogue of this coenzyme was prepared and characterised. There are several positions in the NAD^+ molecule into which fluorine could be introduced, but, in view of the direct involvement of the nicotinamide moiety in the mechanisms of oxidation/reduction reactions involving NAD^+, it was decided to introduce fluorine, in the form of a trifluoroacetyl group, in place of the amide grouping of the nicotinamide moiety. The first step towards the preparation of this compound, 3-trifluoroacetylpyridine adenine dinucleotide (Fig. 67), involved the synthesis of the pyridine derivative, 3-trifluoroacetylpyridine.

Synthesis of 3-trifluoroacetylpyridine.

Initial attempts to prepare 3-trifluoroacetylpyridine were based on the route shown in figure 68 (a), this method being chosen in preference to the published route (pp 23-24) in order to further investigate the fluorinating action of perchloryl fluoride which had already been used successfully at Bath University for the fluorination of diethyl oxaloacetate. The chosen route (Fig. 68a) involves the precursor ethyl nicotinoylacetate which was prepared by the method of Strong and McElvain (1933) (see experimental method (b)). This method gave a better yield (75%) than did the other methods investigated. The spectroscopic data obtained for both ethyl nicotinoylacetate and its hydrochloride showed that the desired product had been obtained in both cases.

The initial preparation of ethyl difluoronicotinoylacetate proceeded as would be expected from consideration of the mechanism

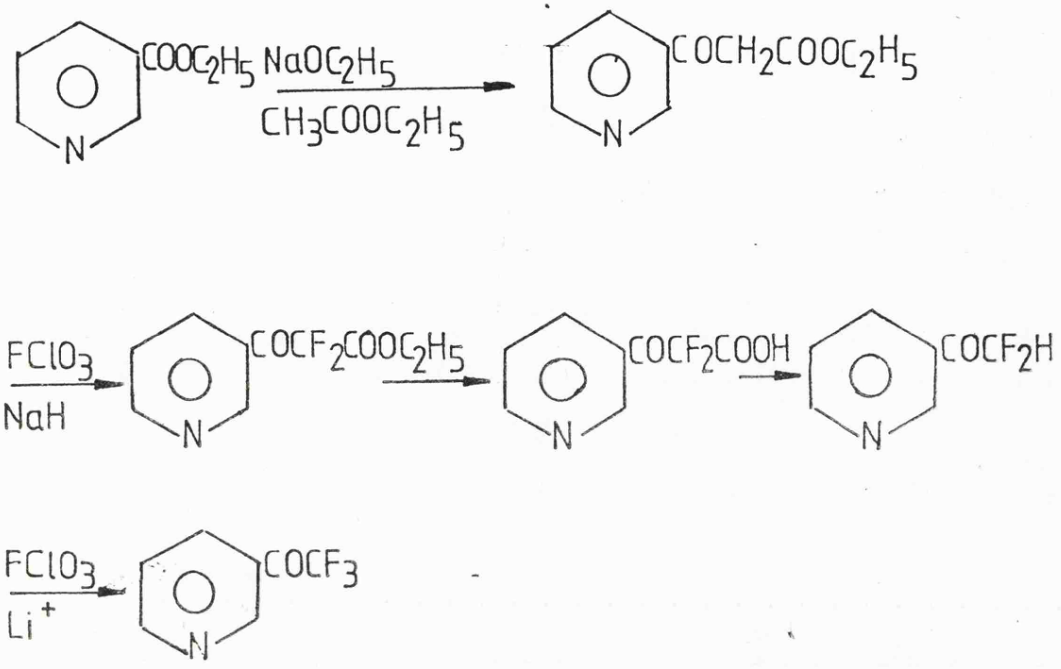
Fig. 67.



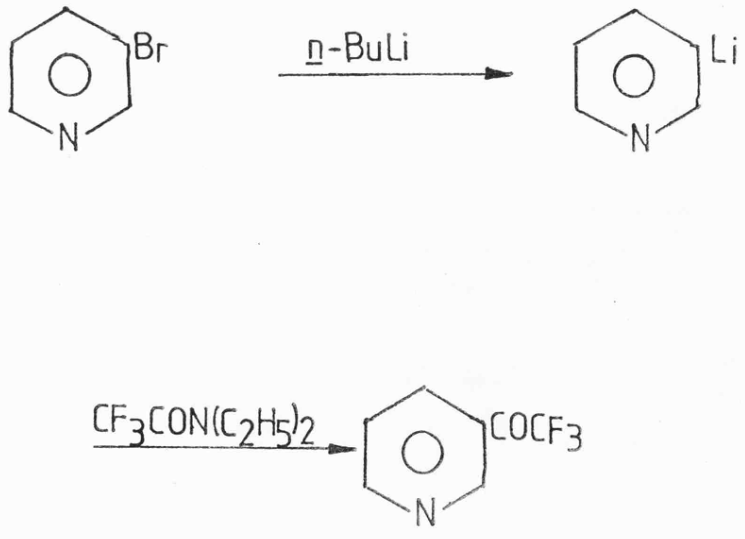
Formula of 3-trifluoroacetylpyridine adenine dinucleotide.

Fig. 68.

Synthesis of 3-trifluoroacetylpyridine.



a) Using perchloryl fluoride.



b) Using the published method.

proposed by Sheppard (1969) (Figs. 14 and 16), which predicts the formation of ethyl fluoronicotinoylacetate, as well as the difluoro compound. Spectroscopic data confirmed that this was the case. Although the initial yield of difluorinated product was, as quoted (p.68), 48%, subsequent attempts to prepare ethyl difluoronicotinoylacetate by this method were less successful, although the fluorination, monitored by g.l.c., did appear to proceed to some extent in most cases. In several attempted fluorinations a peak with the retention time of the compound thought to be the monofluoro compound was noted, and this increased in size with a simultaneous decrease in the size of the peak attributable to the starting material. Strong absorptions at a frequency of between 1100 and 1200 cm^{-1} were noted in the I.R. spectrum of the product from these reactions, these being indicative of the introduction of a $\geq$C—F bond into the starting material. However, a significant amount of the difluoro compound was only obtained in one of the subsequent fluorinations.

It has been found that fluorinations using perchloryl fluoride sometimes do not proceed (G. D. Smith- personal communication), and that the addition of catalytic amounts of water or ethanol have been sufficient to initiate the reaction. Effectively these additions are the replacement of some of the sodium hydride with sodium hydroxide or sodium ethoxide, but in this case it was found that such additions did not cause an increase in the yield of the difluoro compound. The replacement of sodium hydride with n-butyllithium did not lead to the formation of any difluoro compound.

Although perchloryl fluoride is a surprisingly stable molecule, being, for example, thermally stable to 500°C, it is, as well as being a

fluorinating agent, a strong oxidant, and has been known to react explosively with many organic compounds. In one attempted fluorination of ethyl nicotinoylacetate the reaction mixture exploded, and subsequent attempts to prepare 3-trifluoroacetylpyridine by a route involving fluorination by perchloryl fluoride were abandoned for safety reasons.

Preliminary investigations of the further steps involved in the route shown in Fig. 68a indicated that ethyl difluoronicotinoylacetate could be hydrolysed to give the corresponding crystalline acid hydrochloride in 30% yield, but the attempted decarboxylation of the material was unsuccessful. In view of the problems inherent in the preparation of ethyl difluoronicotinoylacetate by the above procedure this was abandoned in favour of the alternative approach to 3-trifluoroacetylpyridine (Fig. 68b) described by Salvador and Saucier (1977).

The preparation of N,N-diethyltrifluoroacetamide and of the crystalline 3-trifluoroacetylpyridine gem diol proceeded smoothly and with yields of 52% and 60% respectively. It is of interest to note that the CH_3 groupings of ethyl groups of N,N-diethyltrifluoroacetamide are in non-identical environments (n.m.r.) presumably as a result of the inhibited rotation of the $-\overset{O}{\overset{\|}{C}}-N$ bond.

The 2,4-dinitrophenylhydrazone of 3-trifluoroacetylpyridine was prepared and was found to have a λ_{max} of 375nm. This compound was subsequently used to estimate the 3-trifluoroacetylpyridine content of the NAD^+ and NMN analogues.

Incorporation of 3-trifluoroacetylpyridine into NAD^+ and NMN analogues.

Both chemical and enzymic approaches to the incorporation of 3-trifluoroacetylpyridine into NAD^+ and NMN analogues were investigated. Initial studies examined the chemical synthesis of both the natural and fluorinated nucleosides and nucleotides.

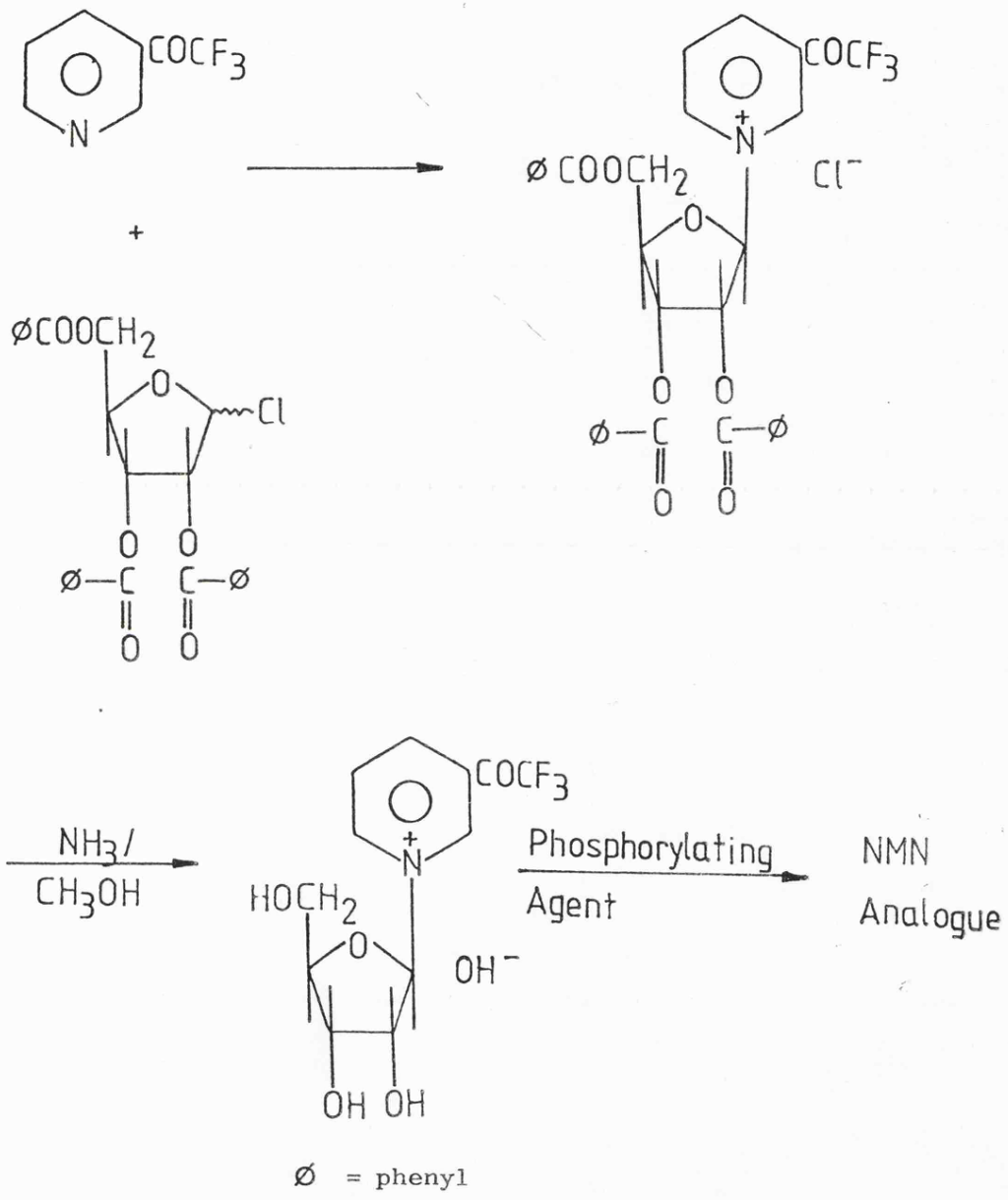
Chemical synthesis of nucleosides and nucleotides.

The preparation of ribofuranosides by chemical means involves the reaction of a base (in this case a substituted pyridine compound) with 1-chloro-2,3,5-tri-O-benzoyl-D-ribofuranose, which is itself prepared from the stable crystalline compound 1-O-acetyl-2,3,5-tri-O-benzoyl-D-ribofuranose (Fig. 69). The synthesis of these materials proceeded as published (see pp 33 to 36)with an overall yield of the chloro compound of 78%. The spectroscopic data of both compounds was consistent with the proposed structures.

^1H N.m.r. spectra of furanoside and pyranoside ring protons generally tend to be complex, containing multiple and overlapping spin-spin splitting patterns. Nevertheless,the chemical shifts of the ring protons of many sugars and nucleosides have been assigned (Varian Associates; Cat. of N.m.r. spectra; 1963), and identification of the signals in the spectra of compounds in the present synthetic sequence was possible by analogy with the published data for similar compounds.

The preparation of the benzoylated ribosides of nicotinamide and 3-trifluoroacetylpyridine was effected with yields of 41% and 42% respectively according to the procedure of Haynes et.al. (1957). In each case nicotinamide(or its analogue) was isolated from the reaction mixture as its hydrochloride, suggesting that some residual HCl remained in the starting material (1-chloro-2,3,5-

Fig. 69.



Ø = phenyl

Proposed route for the chemical synthesis of 3-trifluoroacetylpyridine adenine dinucleotide.

tri-O- benzoyl-D- ribofuranose). As attempts to remove this HCl by the application of a vacuum did not fully succeed, later preparations utilised an excess of 3-trifluoroacetylpyridine, which led to an increase in yield of 18%.

There was some evidence that the products were not stable for an extended period, as evidenced by two peaks in the mass spectrum at m/e = 238 and 236. These peaks are also in the mass spectrum of 1-chloro-2,3,5-tri-O-benzoyl-D- ribofuranose, and appear to be derived from a chlorine containing fragment. It is unlikely that these reflect contamination of the products by the chlorinated starting material, since it is soluble in both chloroform and diethyl ether (the solvents from which the benzoylated nucleosides are purified), and it seems accordingly that the benzoylated nucleosides are not very stable, the glycosidic bond breaking to reform the starting materials. However, storage under refridgeration was found to prevent decomposition. The spectroscopic data, whilst not of a high standard, was generally consistent with the structures proposed for the benzoylated nucleosides.

Debenzoylation of the nicotinamide riboside with a saturated solution of ammonia in methanol proceeded as published (Haynes et. al., 1957), and in a similar yield (70%). The 3-trifluoroacetylpyridine riboside was debenzoylated under the same conditions, but with a much lower yield (8%), which was too low to be of practical use. The use of sodium methoxide or methanolic HCl as reagents for the debenzoylation met with no success, the benzoylated nucleoside being decomposed by both reagents.

Preliminary experiments indicated that the phosphorylation of the debenzoylated nicotinamide nucleoside could be effected by o-phenylene

phosphorochloridate (prepared by the method of Khwaja et.al. (1970) in 42% yield), a compound with the same R_f as NMN being separated by paper chromatography. However, it is possible that the 2' and 3' as well as the 5' phosphate may have been formed as the instability of the glycosidic bond precluded the protection of the 2' and 3'-OH groups with an isopropylidene residue. In view of the difficulties encountered in the preparation of 3-trifluoroacetylpyridine riboside, further experiments on the phosphorylation of nucleosides were not undertaken and attention was turned to the synthesis of NAD^+ analogues by enzymic means.

Transglycosidation reactions involving NAD^+ transglycosidase from pig brain.

In general, the use of NAD^+ transglycosidase from pig brain is the preferred enzymic method for the preparation of analogues of NAD^+ in which the nicotinamide moiety has been replaced by another substituted pyridine compound. The basis of the reaction is shown in figure 70. Factors in its favour are:-

i) the ready availability of pig brain

ii) the ease with which the crude enzyme may be isolated

iii) the stability of the enzyme

iv) the fact that further purification of the crude enzyme is not required

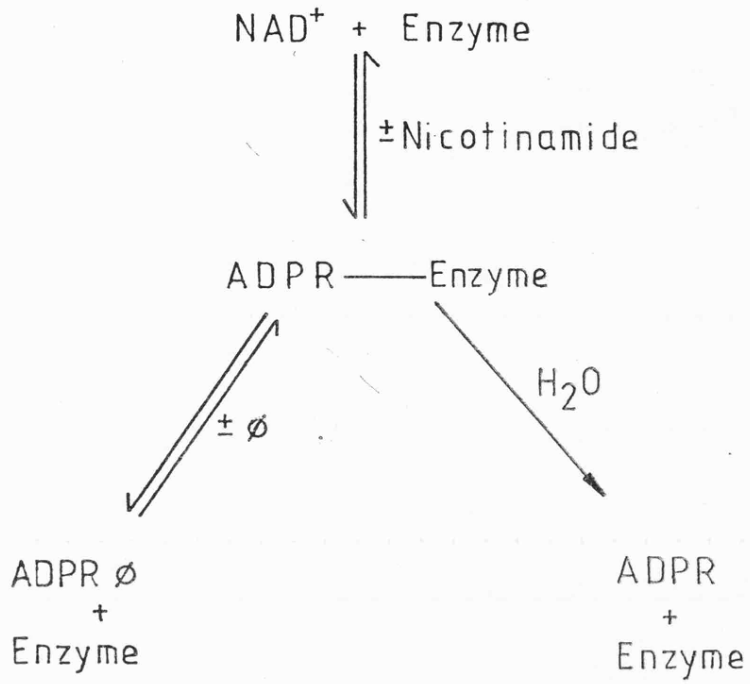
v) the speed of enzymic transglycosidation

vi) the large scale on which the reaction may be performed

vii) the ease of purification and isolation of the product.

NAD^+ transglycosidase was successfully prepared from pig brain by the procedure of Kaplan et. al. (1954), and was used to catalyse the substitution of 3-acetylpyridine for the nicotinamide moiety of NAD^+

Fig. 70.



ø ≡ analogue forming base

Formation of NAD^+ analogues using NAD^+ transglycosidase.

according to the method of Kaplan and Ciotti (1954). The resulting 3-acetylpyridine adenine dinucleotide was obtained as a pale yellow powder in 45% yield.

When 3-trifluoroacetylpyridine was incubated with NAD^+ and the transglycosidase under similar conditions a yellow powder was obtained which did not contain fluorine and behaved chromatographically as ADPR. Reduction of samples of the reaction mixture by ethanol in the presence of yeast alcohol dehydrogenase showed a steady decrease in absorbance at 340nm with time, corresponding to the disappearance of NAD^+ from the reaction mixture. However, in contrast to the corresponding reaction with 3-acetylpyridine no new absorbance attributable to a reduced NAD^+ analogue could be detected. It appears that in the presence of 3-trifluoroacetylpyridine the enzyme behaves simply as a glycohydrolase, and the resulting ADPR is not linked to the fluorinated pyridine derivative. Repeated incubations at a range of pH values similarly failed to yield the required fluorinated analogue. As the latter was subsequently found to be stable under the conditions of the incubation it is unlikely that the analogue had been formed and subsequently hydrolysed in the reaction mixture.

3-Trifluoroacetylpyridine was found to inhibit the transglycosidation of NAD^+ and 3-acetylpyridine, suggesting that 3-trifluoroacetylpyridine might well compete for the active site on the enzyme without having the ability to form a glycosidic linkage. Such behaviour can be rationalised in terms of the negative inductive effect of the 3-trifluoroacetyl grouping.

An alternative approach to the synthesis of a fluorinated NAD^+

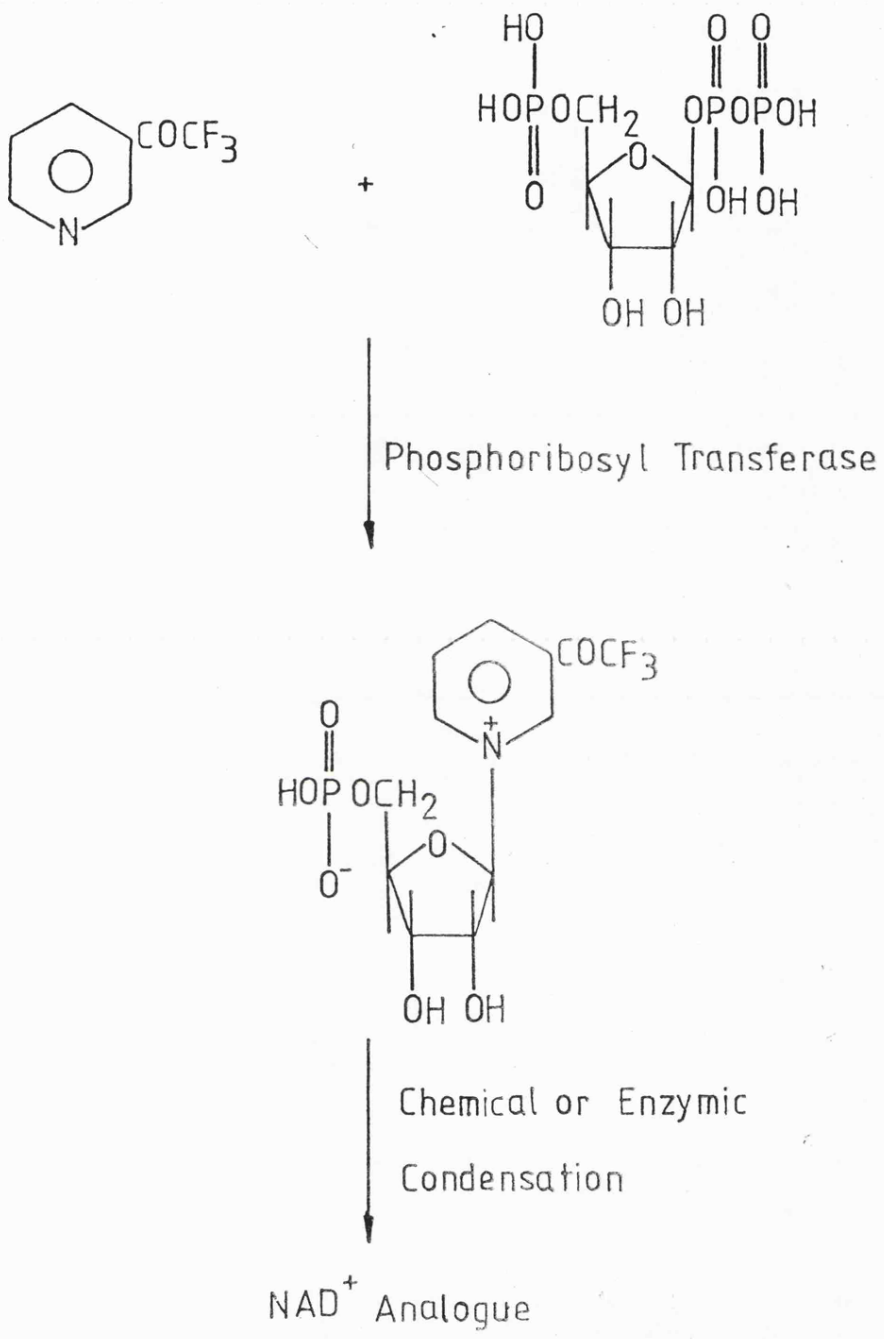
analogue was investigated using NAD^+ transglycosidase in a series of reaction analogous to that used successfully by Biellmann et. al. (1971) in the synthesis of the 3-chloroacetylpyridine analogue of NAD^+. These workers found that the direct incubation of 3-chloroacetylpyridine with NAD^+ in the presence of NAD^+ transglycosidase failed to produce the required NAD^+ analogue, and used instead 3-diazoacetylpyridine in the initial transglycosidation. The resulting 3-diazoacetylpyridine NAD^+ analogue was then converted into the desired monochloro derivative by reaction with chloride ions. It seemed possible that, similarly, reaction of the 3-diazoacetylpyridine analogue with fluoride ions might lead to a monofluorinated analogue of NAD^+. 3-Diazoacetylpyridine adenine dinucleotide was accordingly prepared (in 23% yield) by the procedure of Beillmann and his coworkers. The 3-diazoacetylpyridine analogue was successfully used to prepare the monochloro derivative (73% yield) by the published method, but corresponding incubations with fluoride ions failed to yield a fluorine containing organic product.

In view of the unpromising results obtained using NAD^+ transglycosidase attention was turned to the use of a two stage enzymic preparation of 3-trifluoroacetylpyridine adenine dinucleotide using nicotinamide phosphoribosyl transferase and NAD^+ pyrophosphorylase.

Two-stage enzymic preparation of NAD^+ analogues.

Phosphoribosyl transferase from Lactobacillus fructosus is known to catalyse the transfer of ribose-5-phosphate from PRPP to nicotinamide (Ohtse and Nishizuka, 1967) and the resulting NMN reacts with ATP in the presence of hog liver NAD^+ pyrophosphorylase to give NAD^+ (Fig. 71). The system was accordingly investigated as a route to the preparation of 3-trifluoroacetylpyridine adenine dinucleotide.

Fig. 71.



Synthesis of NAD^+ or a NAD^+ analogue using a phosphoribosyl transferase.

(3-Trifluoroacetylpyridine is used as the substituted pyridine in this example.)

Nicotinamide phosphoribosyl transferase was prepared from Lactobacillus fructosus according to the procedure of Ohtsu and Nishizuka (1967) with a specific activity of 0.009 units mg^{-1} (Ohtsu and Nishizuka quote 0.01 units mg^{-1}). Incubation of 3-trifluoroacetylpyridine with PRPP in the presence of the enzyme led to a fluorine-containing compound with an R_F value (t.l.c.)similar to that of NMN and with a ^{19}F n.m.r. signal having a chemical shift different from that of 3-trifluoroacetylpyridine or its gem diol. Experiments in which 3-trifluoroacetylpyridine was incubated with the enzyme and PRPP in the presence of 7-^{14}C-nicotinamide showed that the fluorinated pyridine analogue did not inhibit incorporation of labelled nicotinamide into NMN to an extent greater than that caused by unlabelled nicotinamide itself.

In view of the apparent success of these preliminary attempts to synthesise 3-trifluoroacetylpyridine mononucleotide, the crude product was incubated with 2-^{3}H-ATP in the presence of hog liver NAD^+ pyrophosphorylase. Thin layer chromatographic analysis of the products showed the presence of two radioactive spots having R_f values consistent with their identification as ATP and the required NAD^+ analogue. The yield of NAD^+ analogue in this reaction was apparently some seven times higher than the corresponding yield of NAD^+ itself when NMN was used as starting material.

The two enzymic steps were combined in an experiment in which 3-trifluoroacetylpyridine was incubated with PRPP and ATP in the presence of both nicotinamide phosphoribosyl transferase and NAD^+ pyrophosphorylase. Again chromatographic evidence indicated that the required NAD^+ analogue had been obtained.

The suspected 3-trifluoroacetylpyridine adenine dinucleotide was then prepared on a 50 μ-mole scale by the single incubation procedure. The product was purified on a Dowex 1X8 column to give a white amorphous powder in an overall yield of 75% from 3-trifluoroacetylpyridine. In view of the similar retention volumes of the product and AMP it was found necessary to destroy the latter compound by the addition of alkaline phosphatase prior to the ion-exchange chromatography stage.

3-Trifluoroacetylpyridine mononucleotide was also prepared on a 50 μ-mole scale, by scaling up the initial incubations in the presence of phosphoribosyl transferase.

Ohtsu and Nishizuka (1967) reported that phosphoribosyl transferase from Lactobacillus fructosus is specific for nicotinamide, and does not utilize nicotinic acid. In view of the apparent activity of 3-trifluoroacetylpyridine as a substrate for this enzyme a number of other 3-substituted pyridine derivatives were tested for substrate activity. From these incubations compounds having similar thin layer chromatographic mobility to NMN were detected when nicotinamide, 3-trifluoroacetylpyridine, 3-acetylpyridine and pyridin-3-al were used as the starting materials. Nicotinic acid, ethyl nicotinoylacetate and 3-bromopyridine, on the other hand, failed to yield the corresponding products.

In the cases when an NMN analogue was detected in the above studies, further incubations in the presence of NAD^+ pyrophosphorylase yielded the corresponding NAD^+ analogues as judged by thin layer chromatography. It appears, therefore, that the combined use of phosphoribosyl

transferase and NAD^+ pyrophosphorylase can serve as a useful alternative to the method using NAD^+ transglycosidase from pig brain in the synthesis of NAD^+ analogues, although the latter method is generally preferred for the reasons already given.

Characterisation of fluorinated analogues of NMN and NAD^+.

The identities of the trifluoroacetylpyridine analogues of NMN and NAD^+ were confirmed in a number of ways. Thus hydrolysis of the glycosidic linkages yielded in each case trifluoroacetylpyridine which was quantified by determination of the absorbance of its 2,4-dinitrophenylhydrazone derivative. Ribose and phosphate contents were measured by standard colourimetric procedures and, in the case of the NAD^+ analogue, adenylic acid was determined by the use of NAD^+ pyrophosphatase followed by 5'-adenylic acid deaminase. In all cases the expected molar contents were found to be present. The homogeneity of the analogues was confirmed by thin layer chromatography.

^{19}F N.m.r. analysis of the NMN and NAD^+ analogues showed in each case a single fluorine signal well resolved from that of the starting material, trifluoroacetylpyridine. The presence of a single peak representing all three fluorine atoms is clearly an advantage in the possible application of the analogue to enzyme active site studies by ^{19}F n.m.r. spectroscopy. While this is the most likely spectrum it is conceivable that the ^{19}F signal could have been split by non-equivalence of the fluorine atoms within the dinucleotide complex and such splitting would lead to diminished sensitivity in its use as a molecular probe.

The fluorinated analogues were found to be similar to the naturally occuring compounds in a number of respects. Thus t.l.c. analysis of 3-trifluoroacetylpyridine adenine dinucleotide in two different

chromatographic system in each case a single zone with R_f value identical to that of NAD^+. Moreover the u.v. spectrum of the NAD^+ analogue showed λ_{max} 259nm and $\varepsilon = 17.5 cm^2$ $mole^{-1}$ compared with λ_{max} 260nm and $\varepsilon = 18.0 cm^2$ $mole^{-1}$ for NAD^+ itself.

The stability of 3-trifluoroacetylpyridine adenine dinucleotide at a range of pH values was investigated by t.l.c. analysis of the radio-labelled analogue. Like NAD^+ itself and many of its derivatives, the fluorinated analogue was found to be increasingly unstable as the pH value of the incubation mixture rose. At pH 9 the analogue was found to have a half life of only 12 minutes. The half life at pH 7 on the other hand was 12 hours which supports the point made earlier that failure of the incubations using pig brain NAD^+ transglycosidase cannot be attriuted to decomposition of the product at the pH value (7.5) employed.

Activity of 3-trifluoroacetylpyridine adenine dinucleotide as an NAD^+ analogue.

Activity as a substrate.

NAD^+ and some of its analogues form addition products with cyanide (fig. 45), having λ_{max} in the 320 to 340 nm region; indeed, this has been used to monitor the extent of the reaction in many transglycosidation reactions using the pig brain enzyme (Biellmann et. al., 1974). The other characteristic reaction of NAD^+ is its reduction to a 1,4-dihydro adduct by reducing agents such as dithionite, bisulphite and cyanoborohydride. However, 3-trifluoroacetylpyridine adenine dinucleotide failed to react with cyanide, nor was it reduced chemically, these judgements being based on u.v. spectroscopy. Examination of the data in figure 45 shows that those analogues that react with cyanide or form 1,4-dihydro adducts

under reducing conditions contain a carbonyl group in the 3-position of the pyridine ring. The effectiveness of the carbonyl grouping in this context can be rationalized in terms of the resonance stabilisation of the addition product. The NMN analogue was also found to be inert under these conditions.

In view of the propensity of α-gem-difluoromethyl ketones, for example difluorooxaloacetate (Kun and Dummel, 1969), to form hydrates and the ability of 3-trifluoroacetylpyridine to exist as a stable hydrate, it seems highly likely that the 3-trifluoroacetylpyridine NAD^+ analogue exists in aqueous solution largely as 3-α,α-dihydroxy-β,β,β-trifluoroethylpyridine adenine dinucleotide. This substituent clearly does not contain a carbonyl group and it may be that this provides an explanation for the lack of reactivity of 3-trifluoroacetylpyridine adenine dinucleotide with either cyanide or cyanoborohydride.

3-Trifluoroacetylpyridine adenine dinucleotide is not reduced by ethanol, malate, lactate or glyceraldehyde-3-phosphate in the presence of the corresponding dehydrogenase. This behaviour is likewise consistent with the existence of the analogue as its hydrate. It may be noted that 3-chloroacetylpyridine adenine dinucleotide is also inert as a coenzyme in several dehydrogenase systems (Beillmann et. al., 1974).

Activity as an inhibitor.

Inhibitors are substances which form enzyme complexes which are catalytically inactive, or less active than the enzyme-substrate complex. If the binding of the inhibitor and substrate to the enzyme is mutually exclusive, the inhibitor is classed as competitive. The structure of a competitive inhibitor usually resembles that of the

substrate, and thus competitive inhibitors are usually assumed to interact with the enzyme at the substrate binding site. In the presence of a competitive inhibitor, at concentration I , the rate of an enzyme catalysed reaction, v_i, is given by:-

$$v_i = \frac{V_m}{\frac{K_m}{S_o}\left[1 + \frac{[I]}{K_i}\right] + 1} \qquad -(1)$$

where, K_i is the dissociation constantof the enzyme-inhibitor complex,

V_m is the maximal velocity,

S_o is the substrate concentration, and

K_m is the Michaelis constant.

Although 3-trifluoroacetylpyridine adenine dinucleotide inhibited the rate of all four dehydrogenases investigated, insufficient kinetic data was obtained to show conclusively that the analogue is a competitive inhibitor of NAD^+ or NADH. However, if the assumption is made that the analogue does act as a competitive inhibitor, which is a reasonable assumption on the basis of the structural similarity, it is possible to estimate the binding constants of the analogue with these enzymes.

For all the enzymes tested the rate equation is:-

$$v_o = \frac{V_m}{\frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A][B]} + 1} \qquad -(2)$$

where, [A] is the concentration of the coenzyme,

[B] is the concentration of the substrate, and

K_A, K_B and K_{AB} are the kinetic parameters. In all the cases

studied the concentration of the substrate, B, was saturating, and thus the second and third terms in the denominator of equation (2) disappears, leaving:-

$$v_o = \frac{K_m}{\frac{K_A}{A} + 1}$$

which is the Michaelis-Menton equation for the cosubstrate, A, with Michaelis constant K_A. In the presence of a competitive inhibitor this becomes:-

$$v_i = \frac{V_m}{\frac{K_A}{A}(1 + \frac{[I]}{K_i}) + 1}$$

following equation (1). The fractional inhibition, i, at a given [A] elicited by a competitive inhibitor is given by:-

$$i = \frac{\frac{[I]}{K_i}}{1 + \frac{[I]}{K_i} + \frac{[A]}{K_A}} \qquad -(3)$$

where, $i = 1 - \frac{v_i}{v_o}$

Solving equation (3) for K_i gives:-

$$K_i = \frac{[I]\ (1 - i)}{i\ (1 + \frac{[A]}{K_A})} \qquad -(4)$$

which allows an estimate for K_i to be obtained. The values found are given in figure 72.

The values of the binding constants are all within the experimentally detectable range of concentrations for ^{19}F n.m.r. spectroscopic investigation using reasonably obtainable enzyme concentrations. This, and the strong resonance singlet of the analogue, makes its use as a

Fig. 72.

Inhibition constants of 3-trifluoroacetylpyridine adenine dinucleotide with various enzymes.

ENZYME	COSUBSTRATE; A	K_A (mM)	i	K_i (mM)
Alcohol dehydrogenase (yeast)	NAD^+; 1.8 mM	0.074	0.11	0.319
Lactate dehydrogenase (hog muscle)	NADH; 0.2 mM	1.07×10^{-2}	0.22	0.18
Glyceraldehyde-3-phosphate dehydrogenase (yeast)	NAD^+; 1.8 mM	0.044	0.09	0.241
Malate dehydrogenase (pig heart mitochondria)	NAD^+; 0.36 mM	0.44	0.4	0.297

tool for investigating the coenzyme binding site of NAD^+ dependent dehydrogenases a promising direction for future studies.

CONCLUSIONS

Conclusions

The use of perchloryl fluoride to prepare ethyl difluoronicotinoyl-acetate, an intermediate in the synthesis of 3-trifluoroacetylpyridine was unsuitable for two reasons. Firstly, the yield from the reaction was variable, and secondly the reaction was found to be hazardous. This method of synthesis had been chosen, in preference to the published method, to further explore the use of perchloryl fluoride as a fluorinatingagent, perchloryl fluoride already having been used at Bath University to prepare diethyl difluoro-oxaloacetate. However, the published method for the synthesis of 3-trifluoroacetylpyridine, involving the reaction of 3-lithiopyridine and N,N-diethyl trifluoroacetamide, was found to be satisfactory.

Chemical nucleoside synthesis was found to be unsatisfactory for several reasons. The method followed was that used in Todd's laboratory and involved the reaction of a substituted pyridine with an acylated haloribofuranose. The number of stages involved in the synthesis is large, and hence the overall yield is poor, and the synthesis lengthy. In the preparation of 3-trifluoroacetylpyridine ribofuranoside the step in which the substituted nucleoside is deacylated proceeded with a yield of only 8%, making the synthesis impractical. The n.m.r. spectra of these compounds was very poor. The signals were very broad and unresolved, this being due, in part, to the insolubility of several of these compounds.

o-Phenylene phosphorochloridate was found to be a suitable phosphorylating agent for the phosphorylation of nicotinamide nucleoside, but the position of phosphorylation was not investigated.

Although NAD^+ transglycosidase from pig brain was found to be a useful enzyme for substituting some pyridine compounds into NAD^+ 3-trifluoroacetylpyridine was unfortunately not a substrate, but was

found to inhibit the incorporation of 3-acetylpyridine into NAD^+. In the presence of 3-trifluoroacetylpyridine and NAD^+ the enzyme acted simply as an NAD^+ glycohydrolase.

Nicotinamide phosphoribosyl transferase from Lactobacillus fructosus was found to be less specific in its base requirements than had been published. The enzyme accepted 3-trifluoroacetylpyridine as a substrate and was not inhibited by this compound. 3-Trifluoroacetylpyridine mononucleotide was isolated from this enzyme system.

Enzymic pyrophosphorylation of the above compound and ATP proceeded to give 3-trifluoroacetylpyridine adenine dinucleotide.

The NAD^+ fluoroanalogue was found to be stable in aqueous solution at pH 5. Neither fluoroanalogue formed an addition product with cyanide, nor were they reduced by cyanoborohydride.

The NAD^+ analogue did not exhibit coenzymic activity with alcohol, lactate, malate or glyceraldehyde-3-phosphate dehydrogenases. It was, however, found to be an inhibitor of all of these enzymes. Approximate values of K_i were determined for the binding of the inhibitor to each enzyme.

Suggestions for further work.

(a) Further and more detailed enzyme kinetic experiments are required in order to determine accurate values of K_i.

(b) $^{19}F_{N.m.r.}$ spectroscopy should be used to investigate the binding of 3-trifluoroacetylpyridine adenine dinucleotide to the enzyme in various dehydrogenase systems, and thus learn more about the active sites of these enzymes.

(c) An investigation into the specificity of NAD^+ kinase with regard to 3-trifluoroacetylpyridine adenine dinucleotide should be undertaken with a view to preparing a fluorinated analogue of $NADP^+$.

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